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CYTOLOGY

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The Problem of Evolutionary Cyto-and Histochemistry

Ya. A. Vinnikov

The Laboratory of Evolutionary Morphology of the Institute of Evolutionary Physiology of the Academy of Sciences USSR, Leningrad

As a result of the fruitful synthesis of morphology, physiology and biochemistry, and, in a number of cases, of biophysics as well, a new biological discipline has arisen at the present time--cyto- and histochemistry, which has been called "The Special Path of Synthetic Biologic Research" (Shabadash, 1949). This discipline permits the posing and resolution of a number of new general biological problems, the solution of which has not been possible for any of the above-listed sciences separately. Cyto- and histochemistry in many instances afford the possibility of detecting the structural distribution of processes occurring in organs, tissues and cells. With the aid of cyto- and histochemical methods, it is possible to determine the presence and distribution of a number of chemically active substances (glycogen, nucleic acids, enzymes, and functional groups), which determine the structure, function and metabolism of the living substrate. Great importance attaches to the efforts now being made to unite cytochemistry and histochemistry with electron microscopy (Barnett and Palade, 1958) and with X-ray structural analysis (Oster, 1957). In this respect, an outstanding example of such a closed circle among morphology, physiology, biochemistry and biophysics, leading to a new synthetic understanding of certain structures, may be seen in the studies of myosine carried out by V. A. Engel'gardt (1941, 1945), A. Szent-Gyorgi (1947) and others; of collagen by A. A. Tustanovskiy and others (1958); of mitochondria by F. Shestrand (1957), O. Lindberg and L. Ermster (1957), A.L. Shavadash (1957) and others; and of desoxyribonucleic acids in connection with the structure of chromosomes by D. Watson and F. Krik (1957).

Even in these examples it can be seen that the study of the special localization of chemically active substances, which are the principal participants in definite energy processes and which determine certain specific functions, is in many cases also the key to an understanding of cellular structure. These chemical substances, especially the enzymes, are strictly adapted to specific structures (Engel'gardt, 1945).

Despite the still limited possibilities of modern cytochemical methods as compared with biochemical methods,

they nevertheless permit in many cases the placing of a bridge between structure and biochemical processes. As is known, biochemical methods also, despite their ready accessibility, suffer from a number of basic shortcomings. One of the most important of these is the necessity of using disrupted organisms, tissues and cells, isolated nuclei, mitochondria, and microsomes which have been isolated with the aid of differential centrifugation. Therefore, only a comparison of biochemical and cytochemical findings will permit a solution of the problem of the localization of one or another biochemical process in definite structures. The existing data indicate that the distribution of chemically active substances depends on the cells of which they are a component part.

It is precisely the different spatial distributions of chemically active substances in living systems which determine the special features of their structure, function and metabolism. In other words, metabolism and energy are apparently regulated by the diversity of spatial distributions of different chemically active substances. In what way does this spatial mechanism evolve in time? The answer to this question, in our opinion, is a problem for evolutionary cyto- and histochemistry. The appearance of chemically active substances and their entry into the composition of structures where they are especially localized, determining structure, function and metabolism, is undoubtedly connected with a prolonged evolutionary process and is determined by the laws of heredity, mutation, and natural selection of organisms. Such an idea follows from the findings of evolutionary morphology (Shimkevich, 1908; Mechnikov, 1917; Severtsov, 1939; Shmal'gauzen, 1940; Zavarzin, 1941, 1945; Khlopin, 1946, and others), of evolutionary physiology (Orbeli, 1942, 1958; Ginetsinskiy, 1945; Koshtoyants, 1945, 1957 and others), and of evolutionary biochemistry (Krebs, 1945, Verzhbinskaya, 1957). Unfortunately, there are still no data on evolutionary biophysics and, in particular, on the evolution of the physical composition and the state of living substances.

The idea that the diversity of spatial distributions of chemically active substances in living substances is determined by the peculiarities of their evolution will be illustrated in a number of examples.

As is known, organisms are divided into aerobic and anaerobic, depending on the character of their metabolism. Among the anaerobic organisms are a few bacteria which do not posesss the ability to utilize oxygen. A considerable majority of organisms belong to the facultative anaerobes, which can live under both aeroboc and anaerobic conditions.

Multicellular organisms, for the most part, are strictly aerobic. However, in the cells of animals, certain processes can nonetheless occur also under anaerobic conditions, at which time energy is derived from glycogenolysis or glycolysis.

Without dwelling on the details of the cycle of glycogenolysis, let us remember only that monosaccharides absorbed from the food are stored in the liver cells, where sugar is converted into glycogen. In the cytoplasm of liver cells, glycogen is localized throughout in the form of luminous microscopic granules. Glycogen is found also in the skeletal musculature, where it is found in a diffuse form in anisotropic discs, (Schubich, 1956). In the photo-receptors of the retina, glycogen is localized in the area of the myoid in the form of granules (Muller, 1926). In the hair cells of the organ of Corti, and in the labyrinttime vestibule, glycogen is distributed throughout the cytoplasm in the form of granules (Vinnikov and Titova, 1957 C). In nerve cells glycogen is connected in the neurons with the Nissl substance, i.e. with ribonucleic acid (RNA), forming a "proteinpolysaccharide" complex (Shabadash, 1949).

Particular interest inheres in the fact that, in the nerve cells of the brain of lower vertebrates and also in the retina and the labarynth, the content of glycogen is considerably higher than in warm-blooded animals (Shabadash, 1949; Verzhbinskaya, 1957; Vinnikov and Titova, 1958 E.).

A reduction in the content of glycogen in the brain and sense organs of the higher vertebrates is naturally connected with the considerable development of the circulation of them. However, in the brain of contemporary mammals also, the most ancient part of the brain is distinguished by the highest content of glycogen and, consequently, by a higher level of glycolytic metabolism. In the brain of mammals (rats and rabbits), glycogen is localized in the neurons of the paraventricular zone, primarily in the Cerea postrema, in the wall of the third ventricle and in the aqueduct (Shimizu and Moricaya, 1957). Similarly, there is a preferential localization here of the enzyme phosphorylase which takes part in the catalysis of a reversible reaction: glycogen plus inorganic phosphate $\rightleftarrows$ glucose-1-phosphate. Phosphorylase is localized in the cytoplasm of nerve cells, outside the mitochondria. At least this is what one may conclude from the results of the cytochemical method developed by Shimizu and Okada (1957). Hence, in the brain of mammals, the distribution of glycogen and phosphorylase is strictly connected with its phylogenetically most ancient parts.

The peculiarities of localization and distribution in

mammals of the substrates and enzymes connected with glycogenolysis, as noted by the histochemical methods, are reflected in the phylogenesis of vertebrate animals, as shown when their brains are studied with the aid of quantitative biochemical methods. N. A. Verzhbinskaya (1957) showed that the direction of changes in the level of anaeroboc glycolysis of the brain in the phylogenesis of vertebrates is directly opposite to that of the intensity of respiration. In the brain of cold-blooded vertebrates, anaerobic glycolysis yields a high index. At 37.5 degrees, the Q N_2/CO_2 (the Q N_2/CO_2 is equal to the number of microliters of CO_2 displaced from the bicarbonate of Ringer's solution by acids formed in the process of anaerobic glycolysis, per milliliter of dry weight of brain per hour). In the brain of fish this is 16 to 29; in amphibia, 18 to 26; in turtles and ciliates, 20 to 24; in birds, 8 to 15; and in mammals, 12. Hence, the capacity of brain tissue for anaerobic production of energy is reduced in warm-blooded animals. On the other hand, there is a marked increase in the oxidative metabolism of the brain. For example, according to the findings of N. A. Verzhbinskaya, the Q O_2 of the brain at 20 to 26 degrees in cyclostomata and fish is 1.8 to 5.2; in amphibia, 2.1 to 2.4; and in reptiles, birds, and mammals, 12 to 14.

Hence, both biochemical and histochemical findings confirm the idea of Putter (1905) (cited by N. A. Verzhbinskaya in 1957) that anaerobic metabolism connected with glycogenolysis is the more primitive type of metabolism. In the course of evolution this type has gradually given way to the more perfect oxidative type.

The distribution of substrates and enzymes of oxidative respiration and their localization in the brain tissue confirms this idea and finds its explanation in it. We shall not dwell on the well-known details of oxidation of pyruvic acid by means of the Krebs cycle, as it occurs under aerobic conditions. It is sufficient to characterize in this connection the basic enzyme of this cycle--succinic dehydrogenase, which is responsible for the dehydrogenation of succinic acid to fumaric acid.

From the findings of Padykula (1955), Leduc and Wislocki (1952), Rottenburg, Wollman and Seligman (1953), Mustakallio (1954), and Shimizu, Moricava, and Joshiaki (1957), it is possible to gain an idea of the histochemical distribution od succinic dehydrogenase in the brain of laboratory animals (mice, rats, and rabbits). The enzyme is found only in the gray substance and is localized both in nerve cells and in the interstitial substance. Its highest activity is seen in the caudal nuclei of the putamen, the anterior nuclei of the thalamus, the interpeduncular nucleus, and the cor-

tex of the cerebellum. Moderate activity is seen in the neocortex, the hippocampus, the optic thalamus, in the nuclei of the oculomotor nerve, the superior and inferior nuclei of the olive, the dorsal cochlear nucleus, the lateral reticular nucleus, the nucleus of the descending branch of the fifth nerve, and the nuclei of Goll and Burdach. A very slight activity is seen in the supraoptic nuclei of the hypothalmus, and in the central gray substance of the mid-brain.

Hence, it is obvious that the weak activity of succinic dehydrogenase in the para-ventricular structures and the hypothalamic nuclei is accompanied in them, as we have already noticed, by high glycogen storage.

Within the limits of the cell and, in particular, the neuron, the activity of succinic dehydrogenase is strictly confined to the mitochandria. This is evidenced not only by data obtained with the aid of fractional centrifugation (Hogeboom, Snyder and Streibach, (1953), but also by recent histochemical studies for the localiz tion of formazone in the cytoplasmic structures (Vinnikov and Titova, 1958; Kirsen, 1958). As to the localization of other components of the Krebs cycle, there are as yet nu cytochemical data available.

Quantitative biochemical findings on the intensity of the oxidative respiration of various divisions of the brain of vertebrate animals indicate an intensification of it in higher vertebrates. In fish, according to the findings of N. A. Verzhbinskaya (1957), the least activity of respiration is connected with the brain-stem. On the other hand, the cerebellum of fish possesses the highest xidative metabolism, Which sometimes even exceeds the metabolism of the forebrain. This apparently is connected with the fact that, in fish, closure of time connections occurs primarily in the cerebellum (Karamyan, 1956). The forebrain in fish is only responsible for the functions of the higher olfactory center (Cappers, 1921; Vinnikov and Titova, 1957 A). Nonetheless, in the forebrain of fish, as Verzhbinskaya has pointed out, oxidative respiration is rather active. Hence, the biochemical preparation for a higher regulatory function, which occurs upon displacement of all the higher centers of activity of the organism into the forebrain, has already been completed. For the higher nervous activity of the forebrain, the substrate and enzymes of oxidative respiration are already prepared in fish; in amphibia, in which there is displacement of certain higher centers into the forebrain, the intensity of respiration of the forebrain is higher than that of the cerebellum. In reptiles, cortical tissue first appears in the forebrain. Its oxidative respiration is high and considerably exceeds the respiration of the cerebellum

and of the brainstem. In reptiles, the forebrain is biochemically and functionally advanced to the leading position (Verzhbinskaya, 1957). It has not as yet been studied histochemically.

In warm-blooded animals--birds and mammals--the great importance of the forebrain and of its mantle is evidenced in the significant increase in activity of respiration of the cerebral cortex over that of the respiration of other divisions of the brain.

This applies also to the cytochrome system of oxidative respiration and, in particular, to its basic enzyme--cytochrome oxidase, which effectuates the aerobic process with the aid of molecular oxygen. This enzyme has been studied quantitatively for the brain (Verzhbinskaya, 1953, 1954, 1957, 1958). Its localization and distribution in the forebrain have been studied histochemically by Shimizu and Moricava (1957). In general, there is a correspondence between the distribution of this enzyme and that of succinic dehydrogenase. But the activity of cytochrome oxidase in the brain of warm-blooded animals exceeds the activity of succinic dehydrogenase, which, according to Verzhbinskaya, is related to the evolution of the dehydrogenase mechanism of oxidation, with the auto-oxidizable participation of the auto-oxidizable flavin dehydrogenases and the auto-oxidizable cytochrome B, and to the development of the cytochrome system of the higher vertebrates in which all components of the system are subordinated to the cytochrome oxidase activity of the tissue. This mechanism of subordination of a more primitive system to a newer and phylogenetically younger system possessing a high reaction rate is, as L. A. Orbeli (1942) has pointed out, one of the basic laws of the functional evolution of animals. In the cytoplasm, cytochrome oxidase is localized in the mitochondria. Hence, a comparison of the biochemical and cytochemical findings on the presence and distribution of anaerobic glycolysis and oxidative respiration in the brain of vertebrates shows that, in the course of evolution, the latter cycle, which is more advantageous in an energy sense, has gradually replaced the former, which is the more primitive cycle (Verzhbinskaya, 1957). This phenomenon simultaneously conforms to the general progress of structure and activity of the nervous system. However, the cerebral hemispheres, even at definitive stages, retain a limited number of the "ancient" parts which can function under anaerobic conditions.

The evolutionary approach permits us to understand not only the factors of distribution and localization of enzymes within the limits of organs. In some cases, the historical method affords us the possibility also of under-

standing complex mechanisms, which control the distribution of chemically active substances within individual highly specialized cells, such as the photoreceptors of the retina, the receptors of the elements of the olfactory organ, and the comparatively simpler secondary secsory receptors of cells of the internal ear, lateral line organs, and the organs of taste.

As is known, the retina of the eye is distinguished by an unusual intensity of both glycolytic and oxidative processes. In this, anaerobic glycolysis in the retina even exceeds the intensity of oxidative respiration. Very characteristic of the retina is the high content of adenosine, triphosphatase, phosphocreatine, and creatine phosphoferase, which testifies to the presence in it of high energy phosphate compounds (Venkstern, 1949). The photoreceptors of the retina, which are primary sensory cells, have been inherited by vertebrate animals from ancient non-vertebrate ancestors (Vinnikov and Titova, 1957A). They are distinguished by a very complex structure. Their peripheral branches, which have either a rod-shaped or a cone-shaped appearance, are divided into external and internal members. About the internal member there is a contractile substance --myoid, which in the lower vertebrates is responsible for the contraction of the peripheral branches upon exposure to light and relaxation of them in the dark (the retinomotor phenomenon). The central branches terminate in a synapse in the outer retinal layer. Electron microscopic studies of the finer structure of the photoreceptors have shown that the photoreceptors are in, an evolutionary sense, primarily ependymal cells, the peripheral branches of which represent a modified ciliated epithelium. The mitochondria in the inner member are strictly localized in the "ellipsoid". These are found also in the external member, and in the region of the synapse of the central branch (Robertis, 1956). Histochemical studies have shown that glycogen and the enzymes associated with it (phosphoamidase, phosphorylase, alkaline phosphatase, etc.) are strictly localized in the area of the myoid of the internal member. Furthermore, toward the outside there is an area in which ribonucleic acid (RNA) is found, along with acid phosphatase. At the very boundary with the external member, in the area of the "ellipsoid" consisting of an accumulation of mitochondria, succinic dehydrogenase and cytochrome oxidase are to be found. Adjacent to this is a thin zone which shows acetylcholinesterase activity. The external member exhibits a laminated structure and consists of proteins which are rich in SH-groups (Wizlocki and Sidman, 1954; Mazanek and Pliczka, 1955; Eichner, 1958, and others).

Within the photoreceptors during their function (upon exposure to light), no visible changes can be detected on the part of the abovementioned substances. Within the ganglion cells of the retina, V. Ya. Brodskiy and N. V. Nechayeva (1958) have recently discovered quantitative changes in the content of RNA depending on the intensity and duration of illumination. Hence, the distribution of chemically active substances in the photoreceptors exhibits a strict and unique mosaic of localizations which, apparently, explains the nature of the anaerobic and oxidative metabolism and of their energy processes. The latter, without any doubt, are associated with the mechanism of reaction of photoreceptors to light.

As is known, the amount of light energy which is capable of causing stimulation of photoreceptors constitutes only a very few quanta. This high sensitivity is due to the presence of a special chemical material which is capable of absorbing light energy. The photoreceptors contain the pigments rhodopsin and porphyropsin in the rods, and idopsin in the cones. Rhodopsin consists of a carotenoid--retinine, which is bound to a protein, opsin. Upon stimulation of a solution of rhodopsin with light, retinine aldehyde-Vitamin A is reduced to Vitamin A and is split off from the protein. Reduction is accomplished with the aid of succinic dehydrogenase in the presence of its co-enzyme --diphosphopyridine nucleotide. As early as 1936, Mirski (1936) suggested that rhodopsin is denatured under the influence of light. This hyppthesis was confirmed by the works of Wald and Brown (1952). These authors showed that light stimulation is capable of "unmasking" the SH-groups of Rhodopsin.

Hence, under the influence of quanta of energy, energy is absorbed by the pigments of the photoreceptors, which are reduced and freed from combination with opsin. The energy transmitted to the protein causes changes in its structure, as evidenced by the appearance of SH-groups. For further steps in the transmission of stimulation, the photoreceptor utilizes the energy both of anaeroboc glycogenolysis and of oxidative respiration. In this, the processes of resynthesis of these energy substrates apparently occur so quickly that it is not as yet possible to detect the functional changes of their distribution in the cytoplasm of the photoreceptors.

A similar mosaic localization of chemically active substances has been discovered by our associate, A. A. Bronshteyn, in the peripheral branches of the sensory cells, which testifies to the common ancient origin of the primary sensory cells. Both the visual and the olfactory cells

must be regarded as relics, despite their important role in the evolution of the central nervous system, in the composition of which they also develop (Vinnikov and Titova, 1957A).

Completely different laws have been discovered for the secondary sensory cells, or so-called hair-cells. The latter are found only in vertebrate animals, in the ancestors of which (the invertebrate animals) they first appeared in the composition of the lateral line organs (in the Silurian period). The secondary sensory cells are a slightly modified epithelial element of the ciliary type which have developed independently of the nervous system as a part of the so-called placodes (Vinnikov and Titova, 1957A). Electron microscopy has shown that these cells are distinguished by a number of special features which are also characteristic of other receptor elements (Smith and Dempsey, 1957). RNA and mitochondria are distributed uniformly throughout the cytoplasm of these cells.

Histochemical studies of the secondary sensory cells of the inner ear, (the organ of Corti and the vestibular apparatus) in a whole series of vertebrate animals have been made by us, together with L. K. Titova (Vinnikov and Titova, 1957B, C, D, 1958 A, B, C, D, E), which have shown that the distribution and localiz tion in their cytoplasm of chemically active substances are distinguished by diffuseness. Glycogens and the enzymes associated with it are uniformly distributed throughout the cytoplasm. The enzymes of the oxidative cycle--succinic dehydrogenase and cytochrome oxidase--are found in the mitochondria, which are more or less uniformly distributed around the nucleus of the cell and at the periphery of it in the region of the ectoplasm. Alkaline and acid phosphatase activity is to be seen both in the receptor cell and in its hairs. The distribution of these phosphatases, which act at different pH values, is subject to gradient laws. RNA, it would seem, is uniformly distributed throughout the cytoplasm of the hair cells in the form of distinct, fine, rod-shaped structures. This applies also to the distribution of protein and of SH-groups which were studied by us together with Z. A. Yakolev. Acetylcholinesterase is strictly localized to the hairs and to the basal surfaces of the hair cells, in the area of the synaptic terminations of the fibers of the spiral and vestibular ganglia. In the body of the cell there is only a weak and diffuse activity of a non-specific cholinesterase.

The hair cells of the inner ear of lower vertebrates show no particular deviation with respect to the distribution of the above mentioned substances. But they are dis-

tinguished by an uncommon richness in glycogen and the enzymes associated with it and by a weaker activity of oxidative enzymes. Attempts to discover a "sound-sensitive" substance have not been successful.

Functional stress--the effects of sound and of acceleration in rotation--has elicited in all instances a decline in the activity of all of the above-mentioned enzymes, proteins, and functional SH-groups, changes in the distribution of RNA and DNA, and a diffuse distribution of glycogen in the cytoplasm of the hair cells.

It should be pointed out that the hair cells of the organ of Corti have proved to be considerably more sensitive in response to an adequate sound stimulus that the receptor elements of the vestibule. As is known, they respond to waves which are equal in amplitude to the diameter of the hydrogen atom. DeVries (1948) and Fatt and Katz (1950) have pointed out that such minor waves, which are comparable to the threshhold of thermal waves (Brownian movement) and which cause simultaneous stimulation of the hair cells, may be compared with the "spontaneous" impulses which stimulate the threshhold of molecular activity. Nonetheless, despite the higher degree of sensitivity, as compared with the sensitivity of photoreceptors, the hair cells of the inner ear do not have "sound-sensitive" substances in their cytoplasm similar to the light-sensitive substances in photoreceptors. Apparently they are not necessary. The fact is that the receptor cells of the inner ear evolved in the onto- and phylogenesis of the vertebrates at a time when both anaerobic glycolysis and oxidative respiration were already developed to a sufficient degree in them. Chemically-active substances and the energy processes connected with them are distributed and localized uniformly throughout the cell body, and are apparently dispersed uniformly along the entire protein chain which constitutes its molecular cytoplasmic skeleton. In other words, at the present stage in the phylogenesis of the vertebrates, a considerable majority of their organs, tissues, and cells, including such specialized elements as the secondary sensory cells, consist of similar "universal" molecular chains along which there are identical potentialities both for anaeroboc glycolysis and for oxidative respiration. Due to this, there is a special receptor sensitivity and a high specialization for secondary sensory cells which possess huge reserves of chemically active substances, enzymes, and functional H-groups. There is no necessity for their creating additional special energy systems such as we have seen in the case of the photoreceptors. Their functions are completely fulfilled by the existing energy resources of glycogenolysis and oxidative respiration. From

this follows the simple structure of the hair cells, despite their very specialized function. However, the absence of "sound-sensitive" substances is nonetheless reflected in the fact that the energy potentialities of the hair cells are not inexhaustible, as are those of the retina, for all practical purposes. The hair cells in mammals under the influence of sound reveal, even within an hour, a special cycle of morphological, functional, and chemical changes which testify to their exhaustibility. Apparently the same laws are seen in the case of neurons, which are also distinguished by a rich and uniform distribution of chemically active substances. In neurons, the reorganization of the molecular structure in the course of evolution also became so universal that it is capable of accomplishing simultaneously and with equal facility both anaerobic glycolysis and oxidative respiration. As is known, in the neuron, with the exception of acetylcholinesterase which exists in the synaptic surfaces of the system, there is no special "nerve-stimulating" substance. Its function, especially the synthesis of acetylcholine, is carried out by means of energy processes of glycolysis and oxidative respiration.

How, then, shall we explain that, in photoreceptors and in olfactory cells, anaerobic glycolysis and oxidative respiration are spatially distributed and localized in the form of a mosaic, isolated one from another within the limits of their members. We believe that there is a single answer to this. The primary sensory cells, such as the visual and the olfactory cells, evolved at a stage of evolution of the mammals in which their energy processes were being carried out only by means of anaerobic glycogenolysis. Despite this, high capabilities for the reception of light and odors were so important that, very soon in the process of selection, improved morphophysiologic and biochemical structures were created--the primary sensory cells with specialized peripheral branches, which, despite the small energy potentialities of anaerobic glycolysis, must be considered to have been, to a certain degree, adequate for the ancestors of the vertebrate animals for purposes of acquainting themselves with the surrounding environment.

Later, when organisms also effectuated oxidative respiration in order to utilize new energy possibilities, it became necessary for the primary sensory cells to form an "extension" in the form of an external member, in the area of which there is a primary localization of the enzymes and substrates of oxidative respiration. A complete reorganization of cells, such as occurred in other parts of the organism in connection with the high specialization of visual and olfactory elements, was as yet impossible. It may be that

their energy processes are associated for this reason with a number of special pigments, which apparently preceded the appearance of oxidative respiration and testify to the high level of their ancient visual function, so amazingly developed in the course of evolution in the modern higher animals.

Hence, only by means of an analysis and comparison of the historical paths of development of receptor primary sensory cells and "new" secondary sensory cells is it possible to discover the mechanisms which are responsible for the presence and special character of distribution of their chemically active substances, and to understand and unify not only their morphology and physiology but also their cytochemical structure.

The significance of the historical approach to histochemical facts may be demonstrated in yet another example. We, together with L. K. Titova, demonstrated the enzyme acetylcholinesterase in the area of the synapse of the organ of Corti, in the so-called spiral external and internal plexuses.

Acetylcholinesterase was found in the organ of Corti in the area of the inferior and middle helices in the internal and external spiral plexuses, and in the superior helix in the area of the internal spiral plexus. Such an irregular distribution may be associated with the particular efferent nerve endings of the autonomic bundle of Rasmussen distributed in this synapse. The mediator of the transmission of impulses from the efferent fibers in this synapse requires additional study. The histochemical study of synaptic endings in the neurons and in receptor structures of the vestibule (the cristae and maculae throughout the whole series of vertebrates beginning from the cyclostomata) showed that, in the inner ear, afferent impulses are also transmitted with the aid of acetylcholine. In all neurons and synapses where there are no afferent endings of the autonomic bundle, there is an exceptionally high concentration of acetylcholinesterase. This could only be intended for acetylcholine. Consequently, acetylcholinesterase in the organ of Corti must also be for the purpose not only for the efferent but also the afferent terminations.

Hence, comparative histochemocal studies of the distribution of acetylcholinesterase have enabled us to make discoveries concerning the peculiarities of behavior of the nerve impulse in the organ of Corti and in the vestibule of the inner ear.

In conclusion, let us consider one other example, relating to the histochemistry of the excretory system. Upon studying the hyaluronidase activity of urine, A. G. Ginet-

sinskiy and L. N. Ivanova (1958) concluded that the process of facultative reabsorption of water is based on an increase in permeability of the interstitial layers and of the cement epithelium of the distal tubules. They suggested that the antidiuretic hormone (ADH), which stimulates reabsorption, causes the excretion by the cells of the enzyme hyaluronidase, which depolymerizes the hyaluronic acid composing the interstitial cement. In the histochemical work of A. G. Ginetsinskiy, M. G. Zaks and L. K. Titova (1958), this proposition was not only confirmed but also led to a considerable extension of the whole problem.

At the climax of water-diuresis, when the secretion of ADH is reduced, the epithelium of the collecting tubules is composed of prismatic columnar cells which are cemented by interstitial layers containing hyaluronic acid. It is also found in the collagen fibers of the connective tissue ground substance distributed between the tubules. Upon the administration of pituitrin, within thirty minutes after the injection of the hormone, the dimensions of the epithelium of the collecting tubules are considerably reduced due to a process of a process of apocrine secretion, and the intracellular hyaluronic acid, which is contained in the collagen ground substance around the collecting tubules, is depolymerized. Simultaneously, after the injection of pituitrin, numerous spaces appear in the meshes of the intertubular network in the immediate neighborhood of the tubules. There is every reason to believe that these spaces are lymphatic capillaries of the kidneys which are opened up under the influence of ADH and which serve for the withdrawal of water and hence participate in its reabsorption. How, then, in the course of evolution of the vertebrate animals did this mechanism of reabsorption under the influence of the hormone of the posterior lobe of the pituitary (ADH) emerge and develop? Preliminary histochemical studies by U. V. Natochin show that, beginning with the amphibia, visible changes in the collagen ground substance of certain portions of the mesonephros occur under the influence of ADH, giving rise to the possibility of reabsorption of water into the surrounding vessels. It is of interest that, in the amphibia, secretory processes also appeared in the epithelium of different portions of the tubules which are, however, effectuated in accordance with the merocrine type of secretions. In birds, there is a reorganization in the kidneys under the influence of ADH which is highly reminiscent of what occurs in the mammals. Hence, the spatial interaction of the hyaluronic acid substrate of the intercellular cement and the collagen interstitial framework with the enzyme hyaluronidase in the excretory system is regulated by the

action of ADH. It is already well developed in the amphibia. In the course of evolution of the vertebrates, a definite function appeared which is determined by the chemical structure of the kidneys.

We shall not consider here other examples which testify to the fact that the diversity in localization and distribution of chemically active substances, enzymes, and functional groups which have arisen and have become incorporated in the course of evolution of organs, tissues, and cells, permits us to understand their structure and function together with their chemical composition.

Hence, the problem of evolutionary cyto-and histochemistry involves studies of the mechanisms which have given rise to the presence and distribution of chemical substances composing living systems and controlling their metabolism, structure, and function in the course of onto- and phylogenesis.

It seems to us that these facts and ideas fully justify the postulation of the problems of evolutionary cyto- and histochemistry and mark out the future paths of their investigation.

Summary

The spatial disposition and localization of chemically-active substances in cells, tissues, and organs give rise to their specific form, function, and metabolism. The general and specific features of the distribution and localization of chemical substances are determined in each separate instance by the evolutionary process and are subordinate to the basic laws of heredity, mutation, and natural selection. This is shown by comparative biochemical and cyto-chemical studies of the localization and distribution of anaerobic glycogenolysis and oxidative respiration in the brain of vertebrates; by the level of cellular organization as seen in primary sensory cells (photoreceptors of the retina) and in secondary sensory receptors of the hair cells of the inner ear, etc. These examples and the above-expressed ideas illustrate the justification for postulating the problem of evolutionary cyto- and histochemistry.

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The Ultramicroscopic Structure of Cytoplasm of Tissue Cells of Animals as Determined by Electron Microscopy

A. M. Karpas (Leningrad)

During the first stages in the development of electron microscopy (from 1939 to the end of the '40's), it seemed to many histologists and cytologists that the approach to the electron microscopic study of tissue and cellular structures was limited by insuperable obstacles, the principal one of which was thought to be the excessive thickness of tissue slices, which would not permit suitable electron microscopic representations. From 1948 to 1953, these difficulties were overcome. In 1949, Neumann, Borysko and Sverdlov (1949A, B) introduced the idea of immersing objects in a plastic - methacrylate. In 1949 Palade (1952A) proposed using as a fixative a one to two percent solution of osmium tetroxide buffered with veronal-acetate. The construction of microtomes was modified and adapted to the new requirements: in 1948, Pease and Baker (1948), in 1950, Hillier and Gettner (1950A, B), in 1953, Sjostrand (1953C), Porter and Blum (1953). In 1950, Latta and Hirtmann (1950) were the first to use glass microtomes; in 1951, Hillier and Sjostrand, and in 1953, Sjostrand, improved the technique of grinding and polishing steel blades to the point of near-perfection.

As a result of all these methodologic innovations it became possible to prepare ultrathin slices with a thickness of 200 to 100 angstrom units, with sufficient preservation of material. Electron micrographs reached a capacity of resolution down to 30 angstrom units; clear representations were obtained of cellular structures and ultrastructures with magnifications from 7,000 to 300,000 times. Later, improvements were made in the methods of fixation and immersion (fixation in isotonic solutions at low temperatures, shortening of the time of fixation, introduction of new fixatives, and the use of the method of freezing and drying of the objects). New ultramicrotomes were introduced, including a microtome with a diamond blade (Fernandez-Moran); thickness of slices was reduced to 50 angstroms; the resolving capacities of the best microscopes were improved to ten to eight angstroms. Sharp electron micrographs of cellular components were obtained with magnifications of 300 to 800 and even 1200 thousand times. In recent years, first class laboratories have been set up in which new cadres of electron microscopists have been trained -- in Switzerland, North and South America, France, Germany, Sweden, Belgium, Japan, and other countries. Studies are being carried out in two direc-

tions: (1) studies in cells and tissues at magnifications from 2000 to 710,000 times, with a gradual transition from the classic histologic pictures to those seen under the electron microscope, and (2) studies of the ultrastructure of cytoplasm at a level approximating the macromolecular (down to 25 to 15 angstroms).

Cytologists and histologists have sometimes expressed doubt as to whether the structures demonstrated with the aid of electron microscopy and the detailed organization of living cells might not in many cases be artefactual. These doubts, of course, are fully justified. However, other ideas are also in order.

Electron microscopy has carefully studied the conditions of preparation of slices and the changes which are elicited in structures and ultrastructures by the individual stages of processing of the material. In connection with this, continuous improvements have been made in all links of the chain of technical processes, and strict rules have been developed for the benefit of investigators. However, many difficulties still remain in the interpretation of the findings of electron microscopy; it requires considerable experience and ingenuity to analyze the specific conditions of each given experiment.

Here is an example of the possibility of a concrete demonstration of the nature of a cellular component: vacuoles found on electron micrographs in cells of the renal tubules turned out in studies made by phase contrast microscopy not to be vacuoles but florescent granules which dissolve in the osmium fixative. This example indicates how necessary it is to use other methods along with electron microscopy.

Considerable importance attaches to electron microscopic studies of one and the same object using different methods of fixation, especially the freezing and drying method along with other methods of fixation. The topography of the components of cells found in electron micrographs, their dimension, the distances between them, and their mutual disposition must be verified by diffraction methods of X-ray investigation at acute angles, and by studies made with the aid of polarizing microscopes. Good agreement of results, as has already been obtained in a number of cases with the simultaneous use of these highly different methods of investigation, testifies to the fact that the structures studied in this way do actually exist. Nonetheless, the threat of obtaining artefacts remains. Reduction in the number of these will always depend on the continuing improvement of techniques, on the care with which the experiment is set up, and on the quality of the interpretation of the represen-

tations obtained.

By now a number of works have been published comprising a tremendous amount of factual material obtained with the aid of electron microscopy. The oldest works are those of Selby (1953) and Sverdlov (1954); more modern materials are those of Selby (1955), Sjostrand (1956), and Bessis (1957). The most profound critical evaluation of the results obtained and of the techniques of investigation is given in the collection of Sjostrand, although it does not pretend to be an exhaustive review and is even partially outdated. A short article devoted to electron microscopy has been published by Frank (1956).

The present article is devoted to the problem of the ultrastructural organization of the "ground substance" of the cytoplasm and of the cellular organoids (I wish to express my heartfelt gratitude to my scientific associate at the Institute of Cytology of the Academy of Sciences USSR, V. F. Mashanskiy, for his comradely assistance in the preparation and reproduction of the figures in this article).

Cellular Organoids

Mitochondria, the Golgi apparatus, and the centriole have been found in all electron microscopic studies of tissue cells. The most extensive studies have been made of the ultrastructure of the mitochondria. On the basis of studies of the numerous electron microscopic pictures made by Sjostrand (1956), a three-dimensional reconstruction of them has been made. Each mitochondrium is covered with a continuous, three-layered "covering" -- an external membrane. Internally the mitochondria are divided into sections by three-layered discs which are perpendicular to its long axis and parallel to each other (Fig. 1). The discs and the external membrane of the mitochondria consist of two extremely dark osmiophilic layers, each of which is 35 to 55 angstroms in thickness and separated by a lighter osmiophobic interval which is 70 to 80 angstroms thick. The dimensions of the components of the mitochondria vary slightly in the cells of different tissues. The double refractility of the intervening layer, established with the aid of polarizing microscopy, together with the above mentioned figures on the thickness of the layers of the membrane and certain related findings, have permitted us to suggest that the osmiophilic layers of the discs consist of oriented proteins and the lighter interval consists of a double layer of molecules of lipids, the long axes of which are parallel to the long axes of the mitochondria. In the mitochondria of different tissues the transverse discs are sometimes larger, sometimes

smaller; sometimes the neighboring transverse discs are joined by segments lying parallel to the long axis of the mitochondria and forming a multiplicity of chambers.

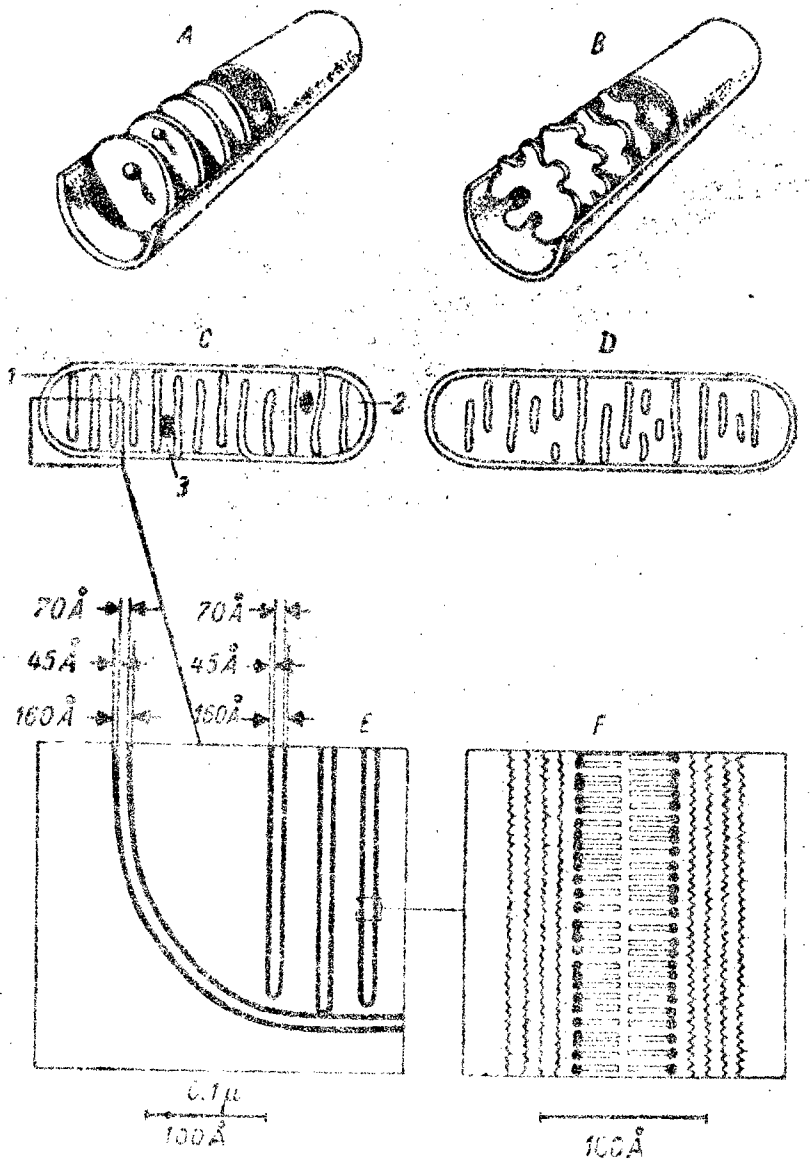

Fig. 1. Three-Dimensional Reconstruction of the Ultrastructural Organization of Mitochondria of Two Types:

A -- Mitochondria from the kidney; B -- Mitochondria from the rods of the retina of the eye; C and D -- representations of thin sections of these; 1 -- external membrane; 2 -- internal transverse disc, 3 -- dark potion of the ground substance; E -- dimensions of the mitochondrial membranes in angstrom units; F -- graphic representation of an attempted interpretation of the ultrastructure of the internal membrane in

"molecular language": Two protein layers divided by a double layer of molecules of lipids. (Sjostrand, 1956).

Palade (1952B, 1953), agreeing with the supposition concerning the existence of the two dark layers of the membrane surrounding the mitochondria, believes that the internal osmiophilic layer dips down in the form of transverse surface accumulations called by him "mitochondrial crests" (cristae mitochondriales), which leave the central space of the mitochondria free. A number of authors have confirmed this conception of Sjostrand in beautiful electron micrographs, whereas other investigators present representations which are similar to the micrographs of Palade. Micrographs of mitochondria havealso been published showing internal concentric layers or tubules. Apparently, careful techniques of study are required (precise orientation of the sections, etc.) in order to be able to judge the true structural organization of mitochondria. It is very likely that their structure changes with changes in the physiologic state, as suggested by Bargmann and others (1955). The internal barriers -- membranes -- are immersed in the ground substance of the mitochondria in which, moreover, dark areas of a rather complex structure can be detected.

As is known, mitochondria contain a rich supply of enzymes. In connection with this, highly speculative theories have been advanced which attempt to explain the ultramicroscopic organization of mitochondria. However, we do not yet have at our disposal sufficient factual material concerning the topography of the enzymes within the mitochondria.

The Golgi apparatus, according to Dalton and Felix (1953, 1954, 1956), Sjostrand and Hanson (1954B, C) Magenau and Bernhard (1955), Afcelius (1956), and others, consists of three components: a membrane, vacuoles, and dark particles (Fig. 2). Double (paired) membranes -- "gamma-cytomembranes" in the terminology of Sjostrand -- are commonly oriented parallel to each other and form a system of three to six or more pairs. The width of each individual strongly-osmiophilic membrane of a single pair is 60 angstrom units. Each pair provides a boundary for a lighter space of 50 to 200 angstrom units in width and forms something akin to a flat saccule. Sometimes this space is distended to the size of a large vacuole. The distance between paired membranes is rather constant -- 60 angstrom units. The membranes are immersed in the ground substance, and are ordinarily homogeneous and finely granular. In the exocrine cells of the pancreas, and in certain other cells, the ground substance of the Golgi zone has been found to contain a large number of granules of differing density, shape, and size which adhere

very closely to the membranes. It is possible also to discover every transitional form from very fine granules to very large, dense zymogenic granules. In the cells of the renal tubules, these granules either do not exist or are present in only small numbers. Large vacuoles surrounded by Golgi membranes are found in the intestinal epithelium. They sometimes contain dense osmiophilic particles. Similar vacuoles could not be found either in the cells of the renal tubules, in the corneal epithelium, in ganglion cells, or in muscle fibers.

Fig. 2. Golgi Apparatus in an Exocrine Cell of the Pancreas of a Mouse.

The paired Golgi membranes, or gamma-cytomembranes (a) are fused at their edges and define a narrow space which sometimes attains the dimensions of a vacuole (b); the surface of the gamma-cytomembrane is smooth. (Compare with the alpha-cytomembrane covered over its external surface by small osmiophilic particles, as shown in Figures 11 and 12); c —

vacuolar spaces surrounded by gamma-cytomembranes; d -- small granules. (Magnification 150,000) (Sjostrand and Hanzon, 1954B).

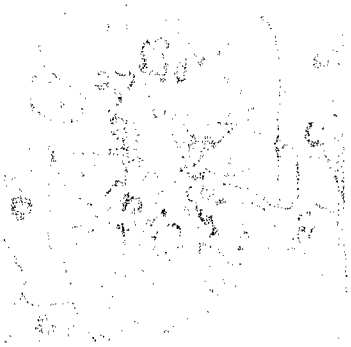

Fig. 3. Cross-Section of a Renal Tubule with Mesodermal and Ectodermal Layers in the Larva of the Snail. Osmic Preparation, under Light Microscope (drawing).

1 -- the Golgi apparatus, 2 -- the nucleus (Hirschler, 1918).

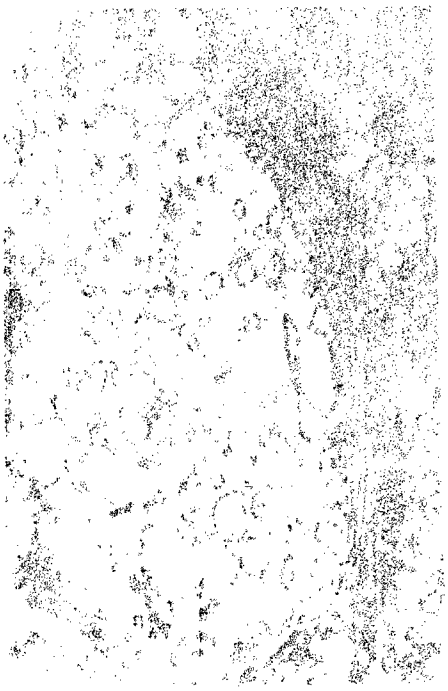

Fig. 4. A Part of A First-order Spermatocyte of the Japanese Snail, Viviparus; Ultrathin Section Through the Golgi Apparatus.

a -- parallel submicroscopic osmiophilic membranes (compare with similar structures in Fig. 3), b -- lighter vacuolar zone (Polister, 1957).

In the auditory sensory (hair) cells of the organ of Corti, membranes have been found which are identical with the gamma-cytomembranes; these form the so-called Hanzon's corpuscles. The most important and unique components of the Golgi apparatus are the gamma-cytomembranes, since they have been found in all cells studied up to this time. Vacuoles and dark particles or granules, as indicated above, may be absent or may vary considerably in shape, size, and number. The Golgi apparatus is very resistant to destructive and mechanical influences. This constitutes one of the evidences that its organization is preformed in the living cell.

There is a complete correspondence between the picture described in the old works of Hirschler (1918; Fig. 3), Nasonov (1923, 1924, 1926) and others, and the modern ideas obtained from studies performed intravitally and under the electron microscope (Fig. 4). The function of the Golgi apparatus has not as yet been clarified. Its topographical connection with the secretory granules, as shown by Nasonov, has been confirmed. However, there have been no studies of its morphologic changes over periods of time and the course of these changes in connection with physiologic influences. For an explanation of this important problem the combined efforts of physiologists, biochemists, and electron microscopists are needed.

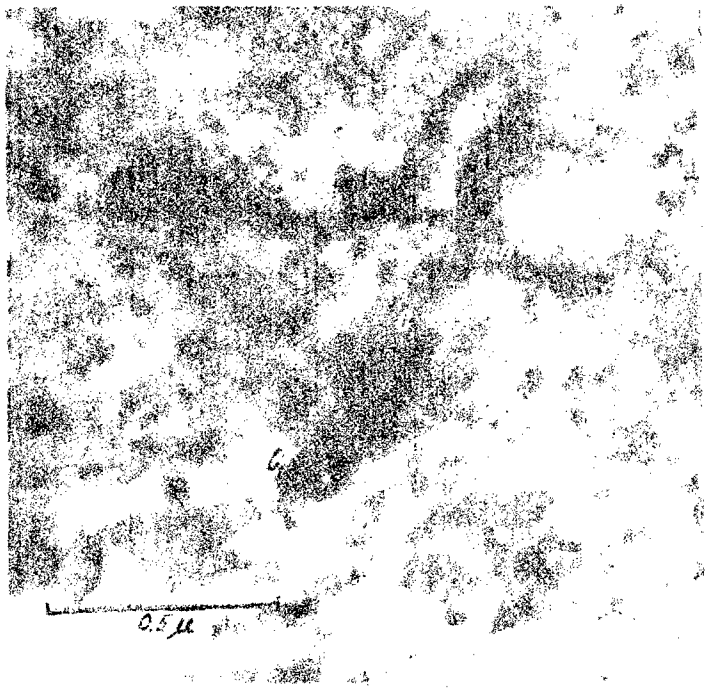

Fig. 5. Cells of the Spleen of a 13-Day Chick Embryo, Probably at the End of the Telophase.

Near the nuclear membrane (mn) -- are two centrioles (diplosomes): C and C_1; C represents a cross section, C_1 represents a tangential section. Magnitication 74,000 (Bernhard and Harven, 1956).

A study of the ultrastructure of the centriole has been made in a few electron microscopic studies. Particular attention is merited by the work carried out in great detail by Harven and Bernhard (1956). In the cells of different vertebrates -- the triton, the embryo chick, the

young chick, the mouse, and the rat -- centrioles are similar in size and shape (Figures 5 and 6). The structure of the centriole is that of a very small, hollow cylinder with a diameter of about 150 millimicrons and a length of 300 to 500 millimicrons. Its highly osmiophilic wall consists of several (9 to 12) fine tubes, (cross-section of each is about 200 angstrom units), disposed in parallel with each other and with the long axis of the cylinder. As early as the end of the last century, Heneguy (1897) suggested that the basal granules of cilia derive from centrioles. Electron microscopy has completely confirmed the striking similarity of the ultrastructures of basal granules of ciliated epithelium (Fawcett, 1955). In connection with this, Harven and Bernhard believe that the elaboration of filiform ultrastructures by different cells (the threads of cilia, the threads of achromatinic spindles, and the threads of spermatozoids) is associated with the presence in these cells of similar ultrastructural formations.

Bessis and Breton-Gorius (1957), studying mature Leukocytes in vivo and under the electron microscope, demonstrated the presence in them of one or two centrioles which were situated in the center of a sphere surrounded by the Golgi apparatus. This centriole also has the shape of an osimophilic cylinder, the wall of which is composed of nine tubes.

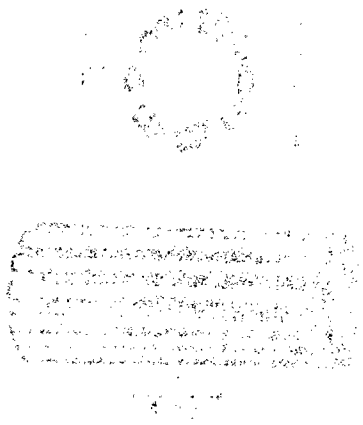

Fig. 6. Three-Dimensional Reconstruction of the Centriole. Above -- a cross-section of it. (Bernhard and Harven, 1956).

The Ground Substance of the Cytoplasm

The ground substance of the cytoplasm of every tissue cell is confined within a very fine, dark, osmiophilic film of the order of 50 to 60 angstrom units, which is the cell membrane. Between cells, there is a light interval which has a uniform thickness of 110 to 130 angstrom units. It is suggested that this is composed either of organized lipoids or of cement substance (Sjostrand and Rhodin, 1953; Sjostrand and Hanzon, 1954A).

Let us review several variants of the cell membrane:

1. The brush border of the cells of the intestinal epithelium is formed of cylindrical processes of cytoplasm (Zetterqvist, 1956). The cell membrane which covers these

processes without interruption and the basic cytoplasm between them consists of three layers: between two outer osmiophilic layers (40 angstrom units each) there is a light interval of 25 angstroms' thickness. From Preliminary findings it appears that, during absorption of food, the character of the membrane changes slightly: instead of two osmiophilic layers only one layer is visible. No pores in this membrane have been detected.

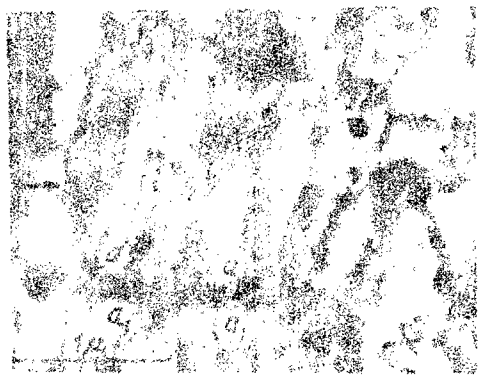

Fig. 7. Longitudinal Section Through Heart Muscle. Arrow denotes the "intercalary band": Light interval (150 to 200 angstrom units) between two adjacent cells is bounded by osmiophilic cell membranes, contiguous with which are dark, dense films of cytoplasm (a and a_1) of each cell. Myofibrils do not penetrate the light intervals. Magnification 48,000 (Sjostrand and Anderson, 1954).

2. The problem of the structure and significance of the intercalary bands of the myocardium remains open to discussion. Some authors are inclined to regard the territory divided by the intercalary band as separate cells. However, it has become accepted to believe that myofibrils pass without interruption from one territory into the territory of the adjacent "cell" through the intercalary band. Electron microscopic studies of the myocardium have enabled Sjostrand and Anderson (1954) to solve this problem. (Fig. 7): the intercalary band is formed by two cell membranes of two adjacent cells, separated by a light interval 150 to 200 angstroms in width, through which myofibrils do not pass. In each of the cells, a very dark, wide, specialized portion of the cytoplasm lies adjacent to the cell membrane. These dark potions of two adjacent cells together with their cell membranes correspond to the "intercalary band" seen under the light microscope. Bourne (1953) made histochemical studies of this zone and discovered in it a high metabolic activity. Recently Couteaux and Laurent (1957) provided electron microscopic confirmation of the cellular structure of heart muscle in the eel (Angilla, angilla), despite the absence of intercalary bands in fish.

3. Policard, Collet and Giltaire-Ralyte (1955), in electron microscopic studies of epithelial cells of the small intestine and of the terminal bronchioles, discovered

special features in the construction of their walls: instead of the even lines which we see with light microscopy, at magnifications of 7,000 to 12,000 times it is possible to detect a crooked line created by numerous overlapping and interpenetrating lateral cellular membranes of adjacent cells (Fig. 8). The authors see in this an adaptation which maintains a continuous connection between the cells during the movements of organs.

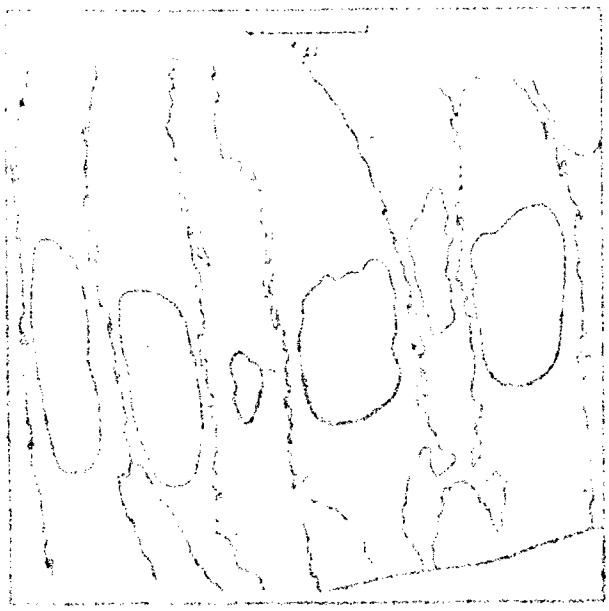

Fig. 8. Crooked Line of the Lateral Cell Membranes of the Epithelium of the Small Intestine. Magnification 7,000 to 12,000 (Policard, Collet and Giltaire-Rallyte, 1955).

4. The myelin sheath of nerves has been shown by the morphogenetic studies of Geren (1954), Geren, Osmond, and Noguera-Graf (1957) to be a spiral which is wound many times around the axis of the cylinder of the nerve in the interval between two nodes of Ranvier. Studies have been made of its early formation as an invagination of the surface layer of the cytoplasm of Schwann cells or other cellular membranes, according to the terminology of Sjostrand. In cross sections it is possible to detect a clear, layered structure, with periods of the order of 120 angstrom units. Dark osmiophilic layers of 25 angstroms thickness alternate with lighter layers, in the middle of which slightly osmiophilic bands can be seen. Recent studies by Fremander - Moran and Finean have not only determined more precisely

the dimensions of all components of the myelin sheath, but have also established, experimentally and visually (Figures 9 and 10), that the dark bands are protein macromolecules, that the light intervals are filled with lipid chains, and that the primary site of precipitation of osmium is probably the point of conjunction between proteins and lipids.

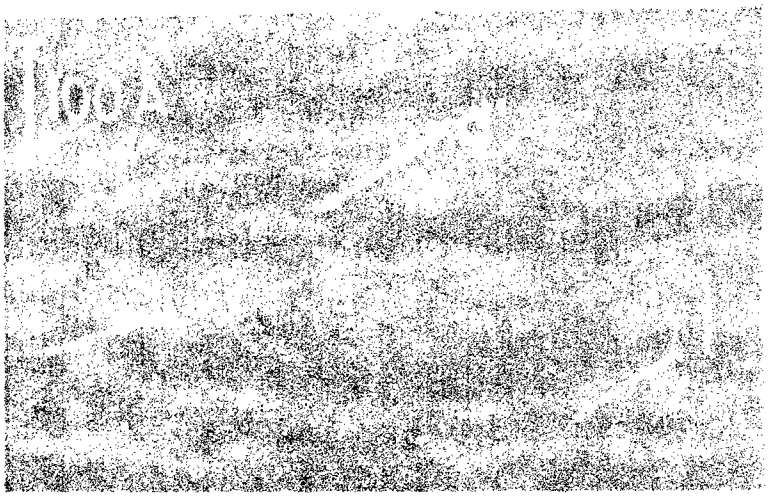

Fig. 9. Cross Section of the Sciatic Nerve of a Rat (Myelin sheath).

Fixation with osmium tetroxide. Dark bands alternate with wide light intervals in which the dark bands (i) are of variable intensity. d' represents a splitting of dark bands. Magnification 1,200,000 (Fernandez-Moran and Finean, 1957).

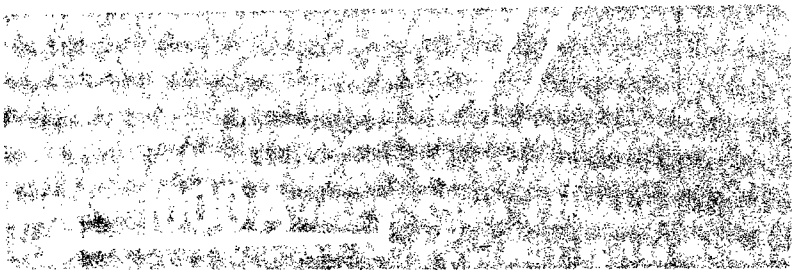

Fig. 10. The Action of Trypsin Over a Period of 12 Hours on the Myelin Sheath of the Sciatic Nerve of a Rat.

Fixation with osmium tetroxide. Attention is called to the degeneration of the black bands into separate, clearly defined granules. Magnification 380,000 (Fernandez-Moran and Finean, 1957).

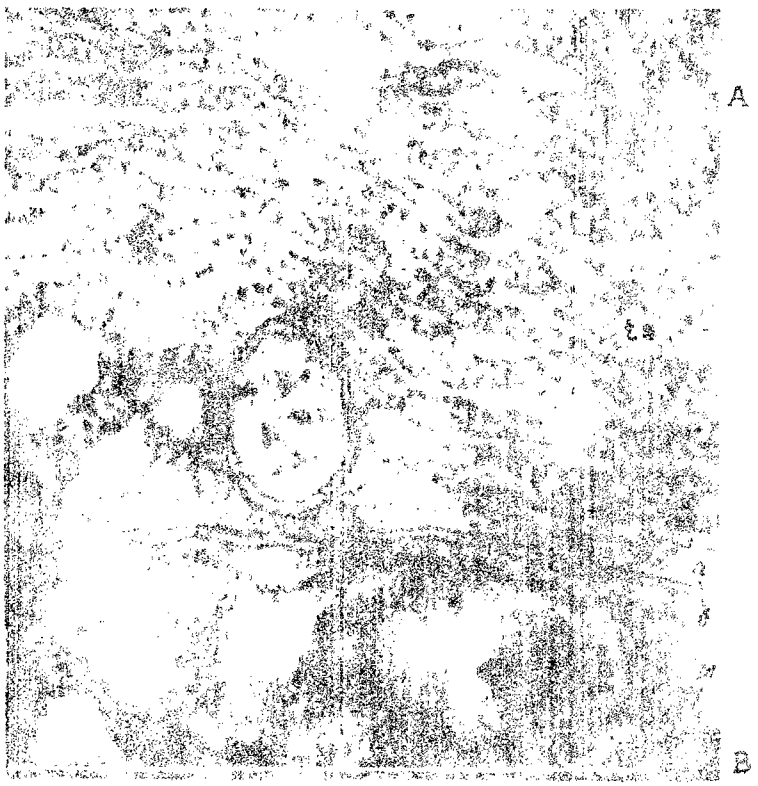

Fig. 11. Parts of Two Adjacent Exocrine Cells of the Pancreas of a Mouse (A and B), Bounded by Two Cell Membranes (a, b) and the Lighter Interval Between Them.

ts -- alpha-cytomembrane of cell A: A formation consisting of two osmiophilic parallel membranes (the thickness of each is 40 angstrom units) separated by a narrow light interval (70 angstrom units), the external surface of which is connected with dark particles (diameter 130 to 150 angstrom units; compare with the gamma-cytomembranes in Fig. 2); M -- Mitochondria of cell B with transverse discs (Sjostrand, 1956).

The ultrastructural organization of the ground substance of the cytoplasm has been studied in detail in the exocrine cells of the pancreas, the cells of the convoluted tubules of the kidneys, and the rods of the retina. The results of these studies are as follows:

The typical exocrine cell of the pancreas of the mouse (Sjostrand and Hansen, 1954A, B, C; Figures 11 and 12) is bounded by the above mentioned cell membrane. In the cytoplasm numerous, very large, osmiophilic, zymogenic granules are distributed at the apex. Large mitochondria are distri-

buted throughout the entire cytoplasm. The Golgi apparatus is between the cell nucleus and the zymogenic granules; all transitional stages can be seen from fine granules adherent to the Golgi membrane to zymogenic granules which are visible under the light microscope. The remaining space in the cell (the ground substance of the ctyoplasm) is filled by the so-called alpha-cytomembranes, which are distributed roughly concentrically around the nucleus. Alpha-cytomembranes are paired. Each of its two component parallel osmiophilic membranes has a thickness of approximately 40 angstrom units; on its external surface, dark clumped particles measuring 130 to 150 angstrom units are either adherent or else are closely bound to it; the internal surface of it is smooth. Each pair of membranes encloses a narrow space of 70 angstroms width, which is less osmiophilic than the membrane. The substance which fills this space is as yet unknown. It may possibly be that this is an organized layer of lipids which is partly dissolved and removed at the time of processing of the material. Hence, each paired alpha-cytomembrane must be regarded as a complex membrane. However, it is also possible to regard the membrane pair as a flat saccule with a liquid content, as has been suggested by Robertson (1954).

Cells of the pancrease have been studied not only in osmium-fixed material but also with the use of the method of freezing-drying in vivo of objects, with subsequent immersion in methacrylate without osmium tetroxide staining. This has permitted demonstration of the same paired dark alpha-cytomembranes. But the dimensions of the space included between the two dark layers of each pair of alpha-cytomembranes has been shown to be considerably narrower and more constant than following fixation with asmium tetroxide, which is probably due to the absence of swelling. The polarizing microscope reveals a doubly refractile cytoplasm in the living cells of the pancreas. The particular features of it are fully consistent with the suggested existence of alpha-cytomembranes. The concordant results obtained with the use of the three different methods in studying one and the same object considerably increase the probability that a membranous organization is inherent in the living cells of the pancreas.

The great number of alpha-cytomembranes in the cells of the pancreas naturally suggests that they participate in the synthesis of secretions. In other cells, alpha-cytomembranes are localized in definite zones of the cytoplasm, such as in the cells of the liver. In neurons they are found in the Nissl substance. They may be completely absent in the rods and cones of the retina of the eye, in cardiac and skeletal muscles, and in the cells of the renal tubules.

They are found in great abundance in secretory organs which elaborate specific proteins: the parotid gland, the liver, the thyroid, the pepsinogenic cells of the stomach, and also in the plasmocytes of the spleen and of the lymph nodes where, according to the ideas of Braunsteiner and Pakesch (1955), the presence of alpha-cytomembranes ("Ergastoplasm") must be associated with the functions of elaborating the specific proteins of blood plasma and of antibodies. Sjostrand believes that the alpha structure of alpha-cytomembranes in all probability "is a useful, organized mechanism for the enzymatically-regulated processes of synthesis" (Sjostrand, 1956, page 494).

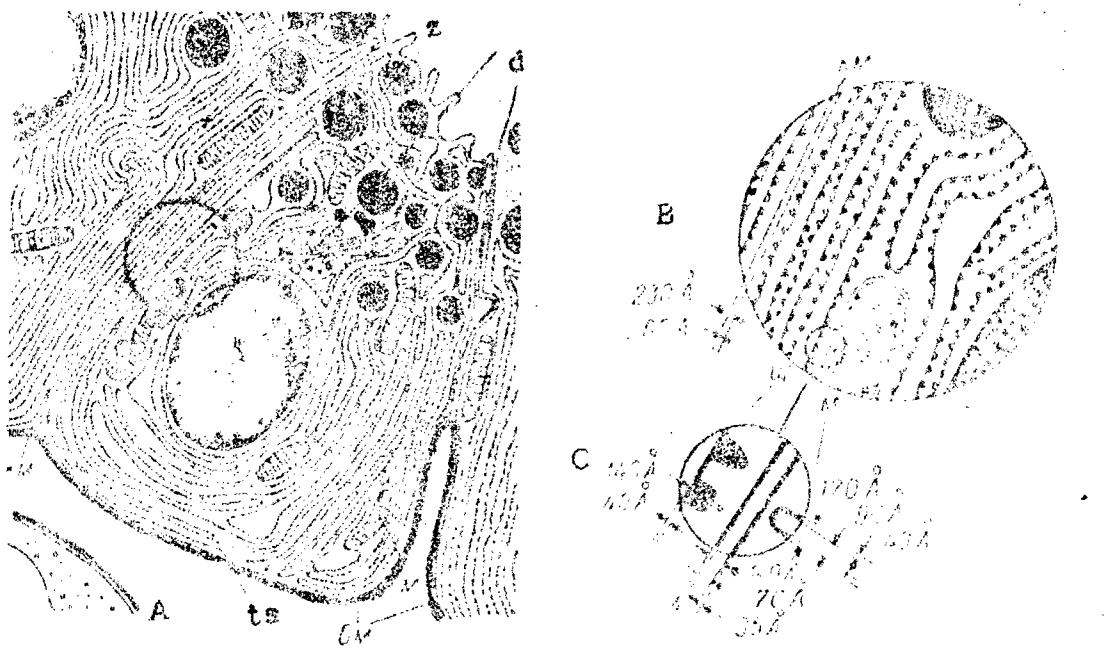

Fig. 12. Representation of An Exocrine Cell of the Pancreas of a Mouse.

A -- general appearance of the cell, z -- zymogenic granules; d -- Golgi apparatus, m -- mitochondria, ts - alpha-cytomembranes, km -- cell membrane of adjacent cells with a light interval, bm -- basal membrane, B -- the portion outlined by a circle in the drawing to the left (A), under higher magnification. Attention is called to the structure of the alpha-cytomembranes, mitochondria, and the size of the components of the cell. C -- portion indicated by a circle in the drawing above (B) (Sjostrand and Hansen, 1954A).

The presence of alpha-cytomembranes in the cytoplasm is often associated with basophilia of the cytoplasm. However, not all basophilia of the cytoplasm is caused by the

presence of alpha-cytomembranes it may also be due to areas containing free, dark particles where alpha-cytomembranes are not found. On the other hand, there are free, dark particles which do not cause basophilia.

Cells of the Proximal Portions of the Convoluted Tubules of the Kidneys

Electron microscopic studies of these cells in mice have enabled Sjostrand and Rhodin (1953) and Rhodin (1954) to demonstrate new microscopic details which had not been demonstrated under light microscope (Fig. 13). On the luminal side, the tubular cells bear a ciliated membrane: the cytoplasmic processes measure 1.4 microns in length and are 500 to 700 angstrom units in width. They are covered with an osmiophilic film of 60 to 80 angstroms thickness. These processes alternate with deep crypts, which are also supplied with a membrane. Beneath the crypts in the ctyoplasm are distributed fluorescing, water-soluble (and hence soluble also in the osmium fixative) granules, which appear on electron micrographs as light staining vesicles. At the base of the cells, the cell membrane intrudes into the cytoplasm in the form of an invagination, called by Sjostrand the beta-cytomembrane, which divides the cytoplasm of this zone into quasi-parallel chambers. Along the beta-cytomembrane, perpendicular to the base of the cell, numerous mitochondria line the chambers. The Golgi membranes (gamma-cytomembranes) and the fine vacuoles associated with them are situated near the nuclear membrane. However, the large vacuoles or vacuolar spaces of the Golgi apparatus, as well as alpha-cytomembranes, are not found here at all. Throughout the cytoplasm are dispersed small groups of fine, dark particles about 50 angstroms in diameter, as well as isolated, intensely osmiophilic, large granules.

All of these factual findings have been confirmed by Bargmann, Knopp, and Schiebler (1955) and Pease (1955). According to Rhodin (1954), variations of the functional state within the limits of physiologic variation do not appear to cause detectable ultrastructural changes in the cells of the proximal convoluted tubules. With "non-physiologic" changes (the administration of egg albumin intraperitoneally in mice), there was a reduction in absorption by these cells, and at the same time, electron micrographs showed disruption of the ultrastructures of mitochondria and fusion of them into large granules. Throughout a certain period of time, it was possible to follow the degeneration of the granules and the restoration of the ultrastructure of the mitochondria. Along with this, there was also a restoration of

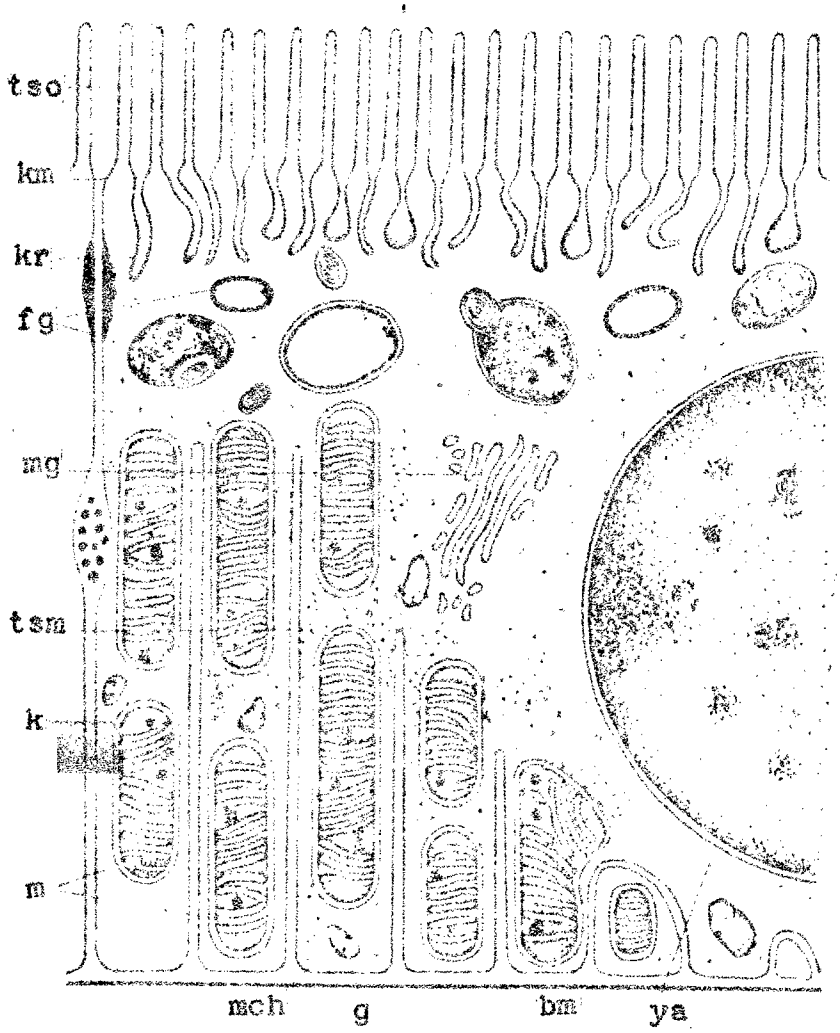

Fig. 13. Schematic Representation of a Cell of a Proximal Convoluted Tubule of the Kidney of a Mouse.

tso -- cytoplasmic processes of the ciliary borders, km -- cell membrane, kr -- crypts, fg -- fluorescing granules, tsm -- beta-cytomembranes, k -- "Chambers," m -- mitochondria, mg -- Golgi membranes (gamma-cytomembranes), mch -- fine, dark particles, g -- large granules, ya -- nucleus, bm -- basal membrane (Sjostrand, 1956).

the normal level of absorption. Farkuhar, Vernier, and Goode (1957), who made microbiopsies of the kidney in cases of human nephrosis, observed in the cytoplasm of the cells of the proximal convoluted tubules under the electron microscope an accumulation of large, dense granules, which corresponded to the "hyaline droplets" of the pathologist. The authors noted every transition from mitochondria to these

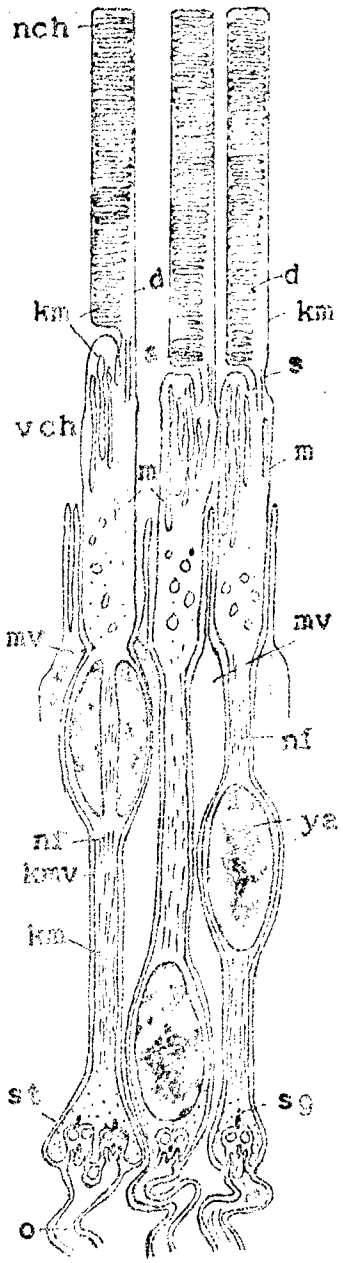

Fig. 14. Schematic Representation of a Rod of the Retina of the Eye of a Guinea Pig. nch - External member, km - cell membrane, d - transverse disc (double osmiophilic membranes), s - fibrillar stalk, vch - internal member, m - mitochondria,

granules. True, they were unable to see a fusion of several disrupted mitochondria into large granules, but they did notice an increase in the size of separate mitochondria, accompanied by changes in their structure. In view of the findings of Rhodin, it may be supposed that this is the result of hyperactivity of mitochondria following penetration into the cells of large amounts of urinary protein, due to injuries of the glomerulus.

The Rods of the Retina of the Eye

A study of the ultrastructure of the retinal rods has been the subject of many investigations, especially the work of Sjostrand (1949, 1953A, B) and Robertis (1956). There is a striking difference between the electron micrograph of retinal rods and the usual representations of rods seen under the light microscope (Fig. 14). In the external member, in addition to the cell membrane already known to us, discovery has been made of a column of transverse circular discs packed densely one on top of another. Each disc consists of a pair of osmiophilic membranes 30 angstroms in thickness each, which by fusing complete a microstaining space 70 to 80 angstroms in width. The distance between the discs is 100 angstrom units. The results of optic polarizing analysis and certain other findings indicate that in the light part of the disc there is a concentra-

nf - neuroprotifibrils, mv - Muller fiber, kmv - cell membrane of the Muller fiber, ya - nucleus of a rod, st - synaptic corpuscle, sg - synaptic granule, o - process of a bipolar cell (Sjostrand, 1956).

tion of lipid molecules, which are disposed parallel to the long axis of the rod. Upon fragmentation of the rod, the osmiophilic membranes may be isolated, which testifies to the stability of their structure and, perhaps, to their protein nature. The above-mentioned dimensions of the components of the external members are in agreement with the results of X-ray structural analysis, which increases the reliability of the electron microscopic measurements.

The external member is joined to the internal member by means of a fine fibrillar stalk. Sjostrand (1956) suggested that this stalk is "to a certain degree similar to the cilia described by Fawcett and Porter." Later studies by Robertis and Franchi (1956) of the structure and morphogenesis of retinal rods (Fig. 15) showed that the entire external member of the rod is like the primitive cilium of an embryonal cell which has grown, expanded, and become modified by the accumulation and differentiation of material which is converted into an accumulation of fine saccules or double membranes. On one edge of the external member, however, nine double fibrils of a primitive cilium are retained and remain connected by short tubules with the double membranes of the disc.

The connecting stalk is the least modified part of the cilium. Its nine double peripheral threads, immersed in the cytoplasm of the internal member, form the basal granule. Both members and the connecting stalk are surrounded by a common continuous cell membrane. In the zone of the basal granule and the zone proximal to it, the internal member is quite literally stuffed with mitochondria, which corresponds to the "ellipsoid" seen under the light microscope. In the proximal zone of the member there are no mitochondria, but there is a Golgi apparatus, vacuoles, dense particles, and neuroprotofibrils. The latter, in their aggregation, form a bundle which emerges from the member in the form of a process directed toward the synaptic granule. This fiber is similar in structure to the fibers of Remak. Its cell membrane is closely adherent to the cell membrane of the lunar fiber (of the corresponding Schwann cell), which likewise forms a three-layered structure (two dark layers separated by a light layer). At the base of the internal member, the zones of cytoplasm adjacent to the cell membranes of the rods and the Muller fibers are much condensed and deeply osmiophilic. Between the cells there remains, as usual, a narrow

light space. However, under light microscopy, the deeply-staining dense cytoplasm of this zone creates the impression of a perforated membrane -- the "membrana limitans externa". In actuality, such a membrane does not exist.

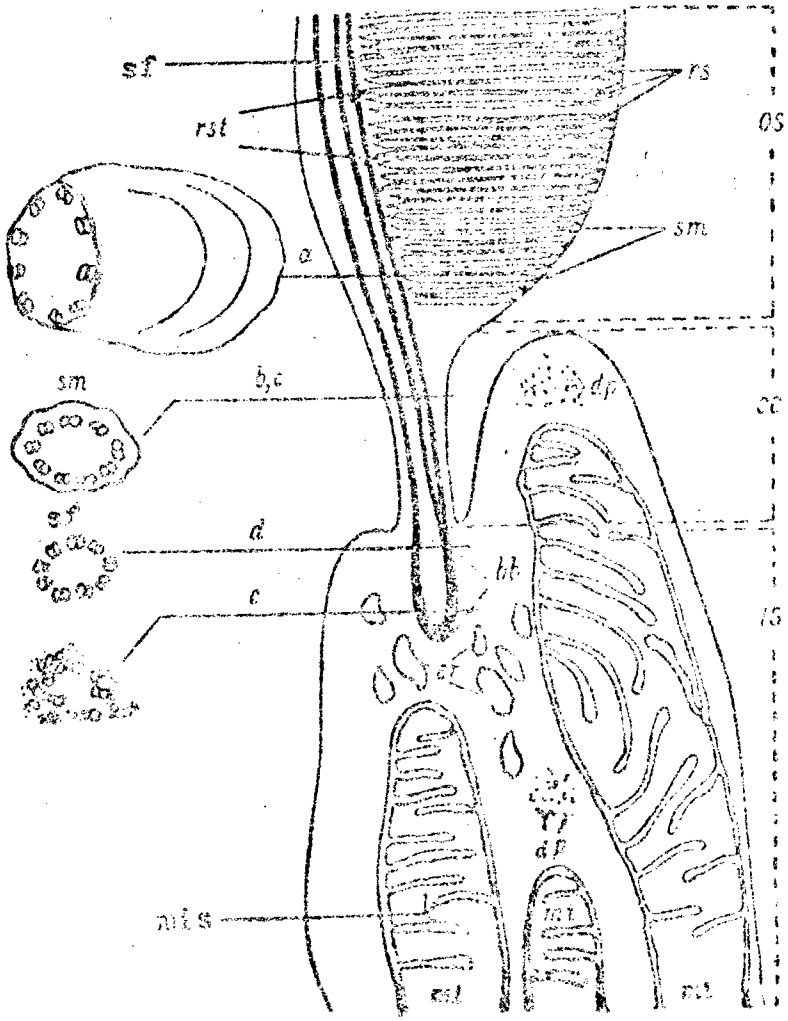

Fig. 15. Scheme of the Submicroscopic Organization of Retinal Rods of the Eye of a Rabbit.

OS -- external member, CC -- free part of the "connecting stalk", IS -- distal portion of the internal member, rs -- flat vesicules, sm -- surface membrane, sf -- threads of the cilium, rst -- short tubules, mi -- mitochondria, mis -- cresta -- bb -- basal granule, er -- vacuoles, dp -- dense particles, a, b, c, d, and e -- different levels of the sections (Roberts, 1956).

In the synapses of the rods there are special gran-

ules with a diameter of 200 to 650 angstroms, which are found in other synapses. These are apparently specific components of the cytoplasm of all synapses. The ultrastructural organization of the rods, as demonstrated by electron microscopy, has led to different interpretations. One of these, as proposed by Sjostrand, is as follows: in accordance with the views of Ingelstam (1955), the external member, with its numerous mitochondria, may perhaps generate the additional e ergy necessary for stimulation of the cell, and thus plays the role of an amplifier. The state of excitation, or stimulation, is transmitted from the internal member to the synapse, probably in a manner analogous to that in which a wave of stimulation is transmitted along a fiber of Remak.

Here it would be advisable to direct attention to the fact that electron microscopic studies of chloroplasts, carried out by Steinmann and Sjostrand (1954; 1955) with high resolution, have disclosed the phenomenon of advanced convergence. The so-called grains of chloroplast are constructed in a manner similar to the external member of the rod and cone; that is, they consist of columns of paired, fused membranes with regular intervals between them. The periodicity is equal to 250 to 270 angstroms, as has been confirmed by X-ray structural analysis. It is inevitable that one should compare this morphologic parallelism with the fact that rods, cones, and chloroplasts are all receptors of light energy.

* * *

Upon studying the above-described factual findings, one inescapably concludes that the "membranous structures" are natural and widely-distributed components of cytoplasm. They may be found in such parts of the ctyoplasm as the external members of rods and cones or in the convoluted renal tubules. However, membranes such as the ultrastructural elements of the mitochondria or the Golgi apparatus are apparently a part of every cell.

We may say with certainty that the term "membrane" is extremely unsuitable, since it elicits a number of diversified and irrelevant associations. The cytoplasm is represented in this, as it were, as being divided by solid barriers into a multiplicity of chambers. In actuality, however, authors using such terms in the description of ultrastructures intend only to emphasize that, in certain portions of the cytoplasm, there is a strict ordering of the organization of macromolecules in space. In some cases -- in the mitochondria or in the discs of the rods -- this organiza-

tion is probably of a lipoprotein nature. In other portions, the organization may be different. This spatial ordering, it would seem, corresponds to the complex enzymatic control processes which occur in definite zones of the cytoplasm. Ordered protein structures, according to Sjostrand and others, are relatively resistant to various influences. In particular, as is known, mitochondria extracted in vivo, by means of fragmentation and centrifugation of the cells, retain the ability to participate in complex enzymatic processes. However, mitochondria of the renal tubules, in the experiments of Rhodin, under the influence of additional protein entering the cell, change their ultramicroscopic organization, are disrupted, and fuse in "hyaline droplets". At the same time, however, they do not lose their ability to recover their disrupted structures and to restore their disordered functions. Probably, the "membranous organization" of one or another component of the cytoplasm is continually changing in connection with the functional state of the cell at any given moment. There have recently been published several studies which support this idea (Roullier and Gansler, 1955; Schulz, 1956, 1957; Luft and Hechter, 1957, and others).

Unfortunately, biochemists and physiologists are not in sufficiently close contact with electron microscopists, which is partly responsible for the lack of a more complete knowledge of the alpha-, beta-, and gamma-cutomembranes; similarly, the functional connections between mitochondria, alpha-cytomembranes, Golgi apparatus, and zymogenic granules in the secretory cells of the pancreas, etc., have not been elucidated. The morphologic pictures of ultrastructures which have been demonstrated must, however, provide a stimulus to the setting up of physiologic and biochemical experiments for the purpose of clarifying a number of important biologic problems.

The incorrect idea may be obtained that alpha-, beta-, and gamma-cytomembranes are the sole elements of the ground substance of the cytoplasm is constructed according to the membrane type. In actuality, however, these cytomembranes are immersed in a more or less homogeneous substance, which has been called a matrix by many investigators (Policard and Bessis, 1956). Unfortunately, up until the present time, the structure of this matrix has not been studied. Demonstration of the membrane type of structural organization has been facilitated by several circumstances: both by the fact that fibrillar proteins are apparently stable, not being disrupted or being changed only slightly by the rather harsh processing of the material necessary for the preparation of thin sections, and by the fact that they scatter electrons and "stain" intensely with osmium. It is a quite different

matter with the matrix. In all likelikhood, the globular proteins which compose it are less stable and are easily disrupted, and, together with lipids and other substances, are partially extracted during processing with aqueous solutions, alcohols, and acetone. The dense dark particles, which without reason are called by biochemists "microsomes," which are scattered throughout this matrix, are preserved. The "microsomes" represent a collective group consisting of particles which differ in form, size, chemical composition, and number. They still require detailed study in different cells both by biochemical and by morphologic means. The numerous vacuoles which are found may actually be vacuoles in a living cell, such as the pulsatile vacuoles and the vacuoles of pinocytosis seen in blood films (Bessis, 1957), or, on the other hand, may simply testify to the fact that the substance filling them was removed during preparation of the sections. The visible vacuolar structure may also be a reflection of fatal changes. For a detailed study of the ultrastructure of the matrix, new methods of fixation, immersion, and "staining" must be developed.

Let us dwell for a moment on the loose term "endoplasmic network" used by American authors and accepted by certain other authors. This term is applied to very different formations in the cytoplasm -- tubules, canalicular formations, vesicles, vacuoles, alpha-, beta-, and gamma-membranes, and others. Without providing a clear morphologic characterization of these formations in their descriptions of them, their use of this term frequently simply introduces confusion. It seems to me that Sjostrand's point of view is correct: at the present state of our knowledge concerning the ultrastructure of cytoplasm we need a precise, morphologic, quantitative characterization of one or another element, with precise account being taken of its orientation and space. Only such a characterization, with careful and exact studies, can be translated into molecular language.

* * *

What we have found out about the ultramicroscopic organization of cytoplasm is the result of creative thought, definite directiveness, and unrelenting effort on the part of scientists over a period of only a few years. Our knowledge is as yet very scanty. Our techniques are as yet far from perfection. But this in the beginning of our penetration into a new area of knowledge -- the area of study of the ultramicroscopic organization of cells. The rapidity with which electron microscopists have succeeded in overcoming technical obstacles gives us reason to believe that

electron microscopy is a method which, in close coordination with the cytophysiology, biochemistry and biophysics of the cell, will lead in the near future to the discovery of highly important biologic laws.

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Quantitative Cytochemical Studies of Ribonucleic Acid in Different Neurons of the Visual Pathways Upon Exposure to a Light Stimulus

V. Ya. Brodskiy and N. V. Nechayeva

The Laboratory of Cytology of the Institute of Animal Morphology of the Academy of Sciences USSR, Moscow

Studies of changes in the content of ribonucleic acid (RNA) in the neurons upon exposure to adequate stimuli have been made by a number of authors. Studies of changes in the content of RNA arising in the ganglion cells of the retina as the result of light stimulation have led to contreversial results. Some authors (Abelsdorf, 1901; Schupbach, 1906; Erlich and Dische, 1950) did not detect any changes in the amount of RNA upon light and dark adaptations of the retina, whereas others noted a reduction in the amount of basophilic substance following exposure to light (Carlson, 1902), and a third group have described an increase in the amount of RNA in the cytoplasm of gangion cells of the retina upon increasing the time of exposure to a light stimulus (Brattgard, 1951; Bech, 1957; Brodskiy and Nechayeva, 1958).

According to the data of Brattgard (1951), within three hours after placing the animals in darkness, RNA completely disappears from the gangion cells of the retina; according to our own findings (Brodskiy and Nechayeva) and the observations of Bech (1957), in the gangion cells of the retina adapted to darkness a certain amount of RNA is always retained; however, the concentration of it is diminished.

Preceding works have touched on the changes in the amount of RNA upon exposure to light stimulation only in one link of the visual pathway -- the gangion cells. The purpose of the present investigation was to study the changes in the amount of RNA in different neurons of the visual pathway in a state of comparative rest and during intensive functioning of it (in the dark and upon stimulation).

The studies were carried out on cells of the inner nuclear and gangion layers of the retina and the cells of the first granular layer of the visual portion of the midbrain of grass frogs kept in darkness for a period of 48 hours or for different periods of time (5, 10, 40 minutes and 1 or 2 hours) of exposure to continuous light (Yarbus, 1956). The material was fixed with a mixture of alcohol, formalin and acetic acid (1:3:0.3) (Brodskiy, 1956a); it was then immersed in paraffin, and sections of five microns thickness were prepared. The RNA was studied with the aid of ultraviolet microscopy and ctyophotometry (Brodskiy, 1955, 1956b).

The present work is a continuation of the preceding work (Brodskiy and Nechayeva, 1958). The results of the measurements are summarized in Figures 1 and 2.

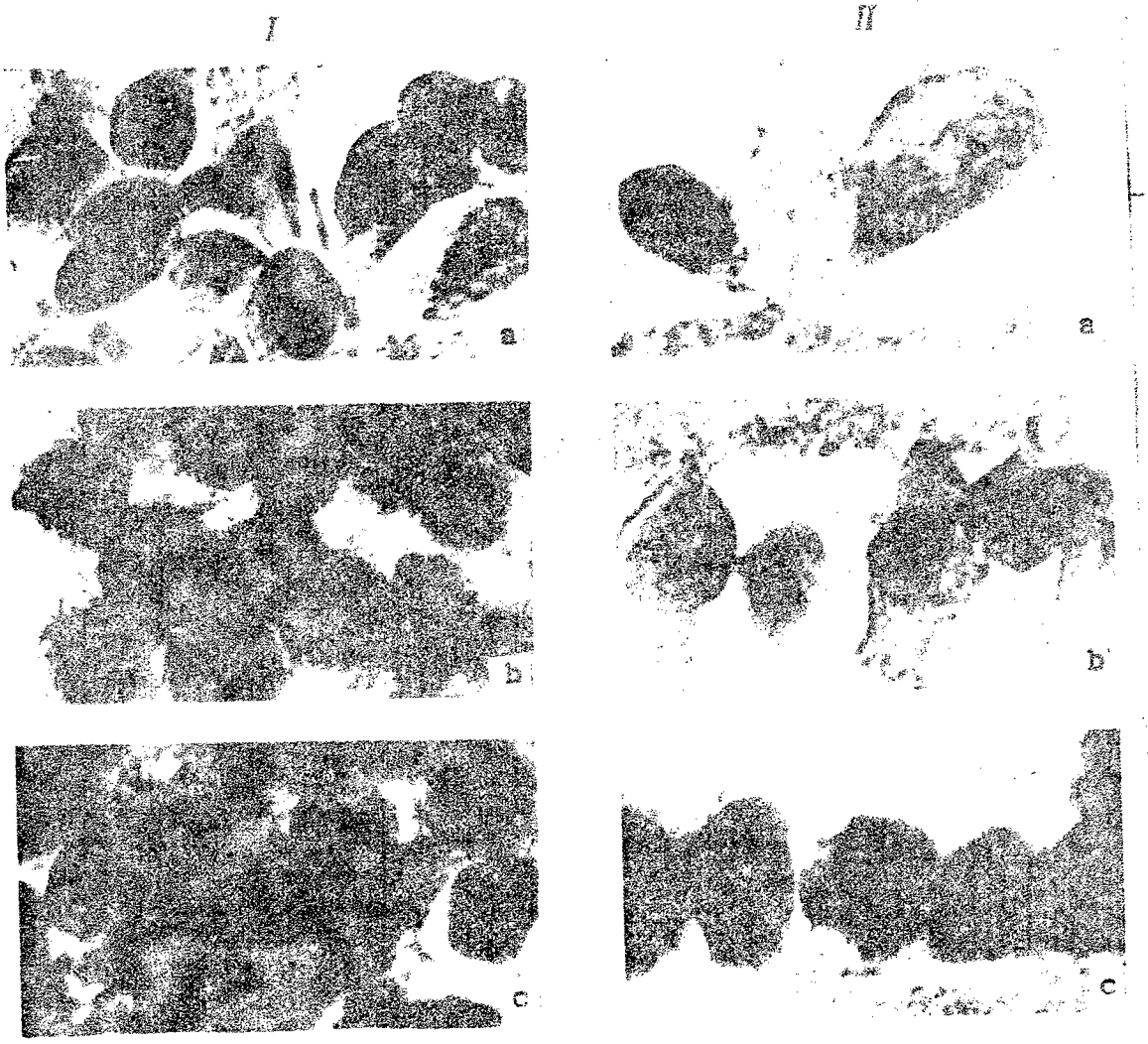

Fig. 1. Nucleic Acid in Different Neurons of the Visual Pathway in Darkness and in Light. Microphotographs with Ultraviolet Rays (265 Millimicrons), Objective 58 Times nA = 0.8, Ocular 8 Times (Magnification 1040).

I -- Bipolar, II -- Gangion Cells. a -- darkness 48 hours, b -- light ten minutes, c -- light two hours for II and one hour for I.

III

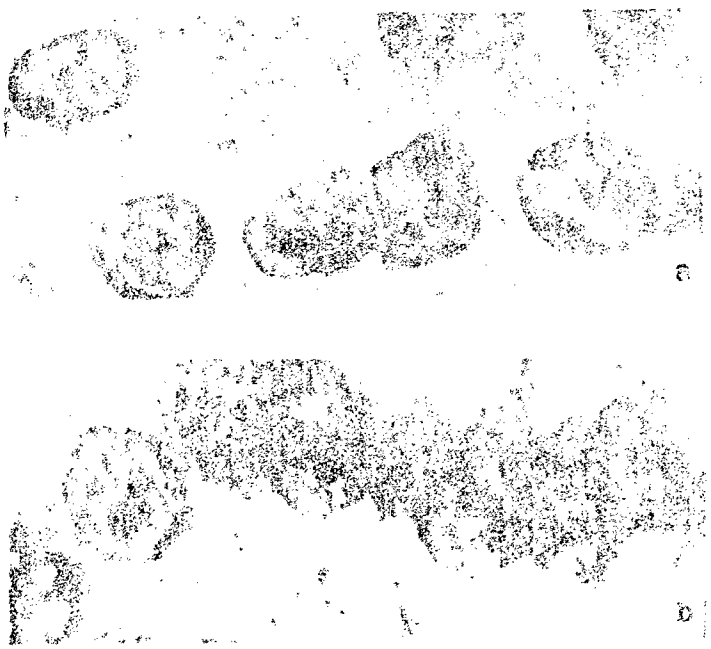

Fig. 1. [continued]

III -- Cells of the first granular layer of the midbrain, a -- darkness 48 hours, b -- light ten minutes, c -- light two hours.

Concentration of RNA in all neurons of the visual pathway in darkness is decreased as compared with the concentration in stimulated cells (subjected to exposure to a continuous light stimulus). However, during the period in which there is almost no visual function (darkness), the three types of neurons studied are characterized by different contents of cytoplasmic RNA. The concentration of RNA in the bipolar cells of retinas adapted to darkness is slightly higher than in the ganglion cells, and in the cells of the first granular layer of the mid-brain it is even higher than in the bipolars. Ten-minute exposure to a light stimulus causes an increase in the concentration of RNA in the neurons of the internal nuclear layer of the retina. The character of the histograms (variation curves) of ganglion cells and cells of the mid-brain remains the same during this time as in darkness. Further exposure to a light stimulus (40 minutes) causes an increase in the amount of RNA not only in the bipolars but also in the ganglion cells. In the cells of the first granular layer of the mid-brain, the optical density of RNA does not change. Upon

exposure to continuous light from forty minutes to two hours, the concentration of RNA increases in all neurons of the visual pathway which we studied: in the bipolars (in the internal nuclear layer of the retina one can distinguish, as is known, three basic types of neurons localized in definite portions of the layers. Thus, the external part of the internal nuclear layer is occupied by horizontal cells, the central part by bipolars, and the inte nal part primarily by amacrine cells. We studied the neurons of the central part of the layer, that is, almost exclusively bipolars), ganglion cells, and cells of the mid-brain.

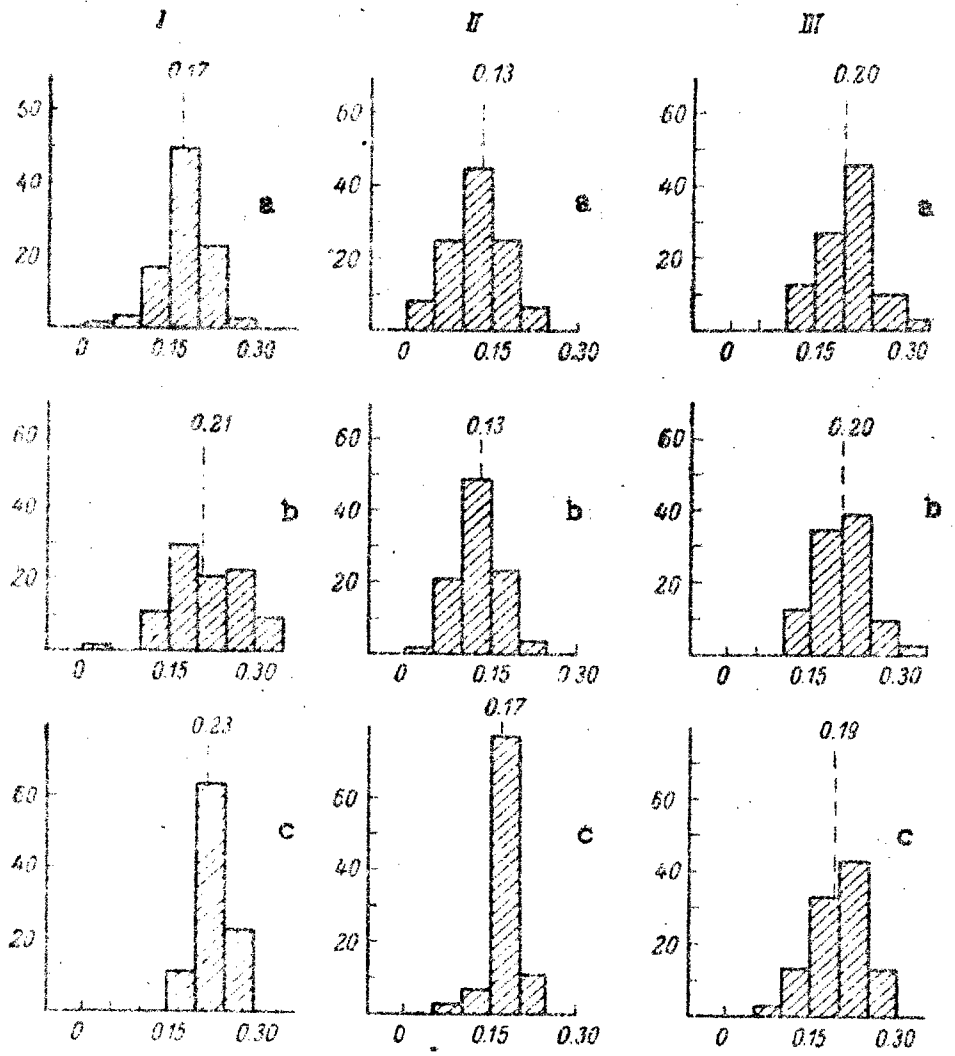

Fig. 2. Graphic Representation of the Changes in Optical Density of RNA in the Cytoplasm of Separate Neurons of the Visual Pathway.

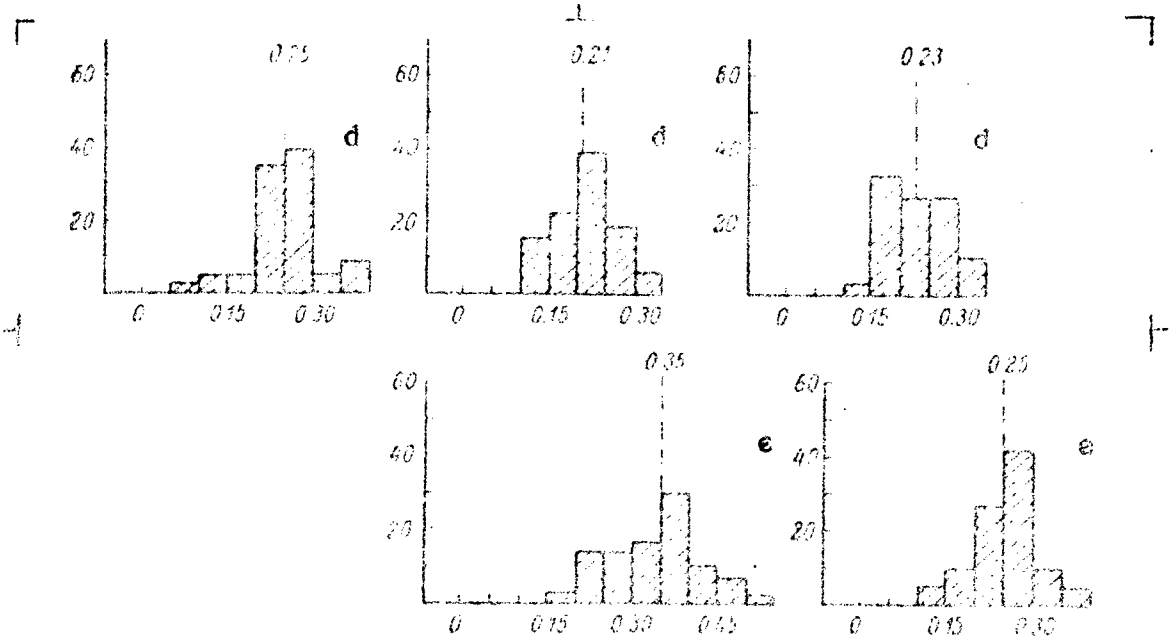

Fig. 2. [continued]

Along the abscissa -- optical density of RNA; along the ordinate -- number of cells in percentages; the average optical densities of RNA are indicated by the dashed lines topped by a number. I -- bipolars; II -- ganglion cells, III -- nerves of the first granular layer of the mid-brain. a -- darkness 48 hours, b -- light ten minutes, c -- light 40 minutes, d -- light one hour, e -- light two hours.

After one hour of illumination, the optical density of RNA in both the bipolar and ganglion cells (in the ganglion layer of the retina, in addition to purely sensory neurons, a certain number of autonomic neurosecretory cells are found. The latter are distinguished from the ganglion cells by the size of the nucleus and by the nuclear-cytoplasmic ratio. Our studies involved only the sensory ganglion cells) increases to the same extent. In the cells of the mid-brain, the amount of RNA begins to increase only after an hour following the onset of illumination, and this increase, as compared with that in the bipolar and ganglion cells, is very slight. Although a two-hour illumination causes an increase in the concentration of RNA in the ganglion cells of the retina of almost three-fold, in the cells of the first granular layer of the mid-brain the amount of RNA during the same period of time increases less than one and a half times.

Our results are in agreement with the findings of Hamberger and Hyden (1949), who observed an increase in

the concentration of RNA in the neurons of the auditory analyzer under normal functional stress and a reduction of it upon exhaustion of the neurons.

As can be seen from the findings which we obtained, a light stimulus causes an increase in the amount of RNA in all of the neurons of the visual pathway which we studied. However, the changes in the concentration of RNA are a consequence and not a cause of the functioning of the neurons. This is indicated by the fact that complete working capacity of nerve cells is observed even in the presence of comparatively small amounts of RNA. The amount of RNA in the neurons of the retina increases considerably later than does the functional activity of these neurons upon exposure to a light stimulus.

An analysis of the histograms characterizing the concentration of RNA in the cytoplasm of neurons indicates that the amount of RNA within a given group of nerve cells varies considerably. Thus, upon adaption of animals to darkness, we found isolated cells in each of the layers of neurons which we studied which contained RNA in amounts equal to that found in elements subjected to light stimulus. The ratio of the number of these cells to the entire number of cells varied in different parts of the visual pathway. In the internal nuclear and ganglion layers of the retina of animals adapted to darkness, about 25 percent of cells contained an increased amount of RNA, and about 50 percent of the cells in the mid-brain contained an increased amount of RNA under the same conditions.

The presence of RNA in nerve cells in states of comparative rest (the retina kept in darkness) may be explained by the fact that the processes of replenishment of proteins in the neurons occur during any of its functional states. Therefore, a certain amount of RNA is retained even in the body of a non-stimulated nerve cell. This minimal content of RNA differs from different types of neuron, for example, the motor neurons of the spinal cord (Brodskiy, 1956a), the bipolar and ganglion cells of the retina, and the cells of the mid-brain. Such differences in the amount of RNA depend, it seems to us, primarily on the total amount of protein in the neurons: the greater the protein content of the nerve cell, the more powerful apparatus for its replenishment it must have. However, this is only one of the possible causes of the preservation of RNA in a non-stimulated nerve cell, although it is probably the most important cause.

It is also quite possible that the differences in the amounts of RNA in the various ganglion cells of the retina in darkness are a consequence of the different spontaneous activity of these neurons, which is already well known in

electrophysiology (Granit, 1947). In particular, this pertains to the neurons of the mid-brain. Thus, according to the data of Jung and Baumgartner (1955), about 50 percent of the nerve cells of the visual cortex of the cat possess a high spontaneous activity and do not react to light. Possibly, it is this heightened, spontaneous activity which explains the high level of RNA in the neurons of the mid-brain of frogs kept in darkness, and the relatively small changes in RNA under conditions of varying stimulation. The spontaneous activity of neurons of the retina, as is known, is not great. A light stimulus causes a considerable increase in the electrical activity of the majority of them. It may be supposed that this explains the great lability of RNA of the neurons of the retina as compared with the elements of the mid-brain.

The above-listed factors, and probably certain other as yet unknown factors, determine the differing chemical characteristics of neurons which differ in their function. The reality of cytochemical differences between sensory and motor nerve cells is indicated by the works of G. I. Roskin and Shornikova (1953) and Roskin, Zhirnova and Shornikova (1954), who studied these cells for the distribution of RNA, DNA, phosphatase, and certain amino acids. L. B. Levin'son and his associates (Levin'son and Ananova, 1956; Levin'son and Leykina, 1956) obtained findings concerning the chemical differences of different neurons in embryonic material.

Upon exposure to a light stimulus, the amount of RNA in different neurons increases to unequal extents. As was noted above, after an hour of illumination with continuous light, about 25 percent of cells of the retina and 50 percent of neurons of the mid-brain generally retain a reduced amount of RNA equal to that which would be expected in elements of animals adapted to darkness. Two to three hours after onset of exposure to a light stimulus, all of the cells of the retina contain an increased amount of RNA; however, the content of this substance varies in different cells. The variability of the concentration of RNA in the cytoplasm of neurons of the retina exposed to a light stimulus is probably explained by the fact that the nerve cells function at different times and with differing intensity. Differences in the changes of RNA between the bipolar and the ganglion cells, on the one hand, and the neurons of the first granular layer of the visual portion of the mid-brain, on the other, may perhaps be explained by different structural and functional (and, as a result, chemical) properties of the neurons at the different levels of the visual pathway.

Conclusions

1. Following light stimulation of different neurons of the visual pathway, the concentration and amount of RNA in the ctyoplasm changes.

2. The content of RNA in unstimulated neurons at different levels of the visual analyzer differs. An increase in the content of RNA in these cells as the result of exposure to a light stimulus begins at different times in different neurons of the internal nuclear and ganglion layers of the retina and the cells of the visual portions of the mid-brain. The intensity of increase and the concentration of RNA differ in different layers.

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A Study of the Granular and Vacuolar Inclusions Arising in Cells Upon the Action of Novocaine and Certain Other Substances

P. P. Rumyantsev

The Laboratory of Cell Adaptations of the Institute of Cytology of the Academy of Sciences USSR, Leningrad

Using the method of Vital Staining, Vol'fenzon (1954) showed that local anesthetics are not, strictly speaking, neurotropic agents since in similar concentrations they cause similar paranecrotic changes both in neurons and in non-neural cells. Vol'fenzon also discovered that novocaine in paranecrotic concentration causes a reversible contraction of fibroblasts which Aleksandrov and Vol'fenzon (1956) compared with muscular contraction and believed to be a consequence of a persisting local stimulation.

Hence, morphologic findings concerning changes in tissues upon exposure to novocaine and to procaine have permitted a new approach to the problem of local anesthesia from the point of view of the most general conceptions concerning functional and substantive changes in stimulated and injured tissues -- the theories of parabiosis of Vvedenskiy (1901) and the denaturation theory of Nasonov and Aleksandrov (1940). These facts are sufficient to indicate the prospects afforded by studies of the effects of pharmacologic agents by cytological methods, especially by means of in vivo microscopy. The latter, however, is at present used quite insufficiently for these purposes, and lags far behind purely physiologic, biochemical and pharmacologic studies of this problem.

Continuing his cytologic studies of the action of local anesthetics, along with his associates, Aleksandrov has discovered that, in cells of the isolated cornea kept in Ringer's solution, following exposure to novocaine, fine non-staining inclusions are formed which are reminiscent of the granules of vital stains. It has been suggested that novocaine, and possibly certain other alkaloids, may to a certain extent behave in a fashion similar to the basic granular dyes. Without regard to the correctness of this supposition, the fact itself of the formation of visible (in vivo) structures upon exposure to novocaine in non-neural cells is of considerable interest and is deserving of analysis, to which the present study has been devoted.

It appears that studies have not been made for comparing the behavior of vital stains with the effects of alkaloids on the cell. However, the possibility has not been excluded that the formation of inclusions, similar to those noted by

Aleksandrov, is what has been seen by authors describing vacuolization of the ctyoplasm of certain protozoa and cells of vertebrates following exposure to cocaine (Schurmayer, 1890; Korentschewsky, 1903; Erlikh, 1911), novocaine (Vol'-fenzon, 1954), morphine, conine, piperidine (Heinz, 1890), and other substances. The majority of the investigators mentioned above have confined themselves to statements concerning the formation of vacuoles and have not attempted to study the course and significance of this process any further.

Heinz crushed erythrocytes of the frog in which there were "vacuoles" which arose following exposure to alkaloids. In this process, the "vacuoles" came out into the physiologic solution but were immiscible with it. On this basis, the author decided that they are rather dense formations. In addition, Heinz applied vital stains to these erythrocytes and discovered accumulations of dye (Bismark brown) in the "vacuoles" and in the nuclei.

Materials and Methods

The present study was rather extensive, due to which the conditions of the experiments, the objects, and the agents tested in this study were rather diversified. Therefore, in the present section, only the most general aspects of the methods of the work will be discussed. Additional descriptions will be given for individual series of experiments. The formation of inclusions upon exposure to novocaine (in the rest of the text, inclusions engendered by exposure to novocaine will, for brevity's sake, be called "novocaine" inclusions, those formed upon exposure to quinine will be called "quinine" inclusions, etc. This does not prejudge the problem of the incorporation of the corresponding alkaloids into the composition of the inclusions themselves) was studied primarily in the epithelium of the cornea and of the skin of the frog, Rana temporaria L., and in the liver cells of the white mouse. The action of other agents (cocaine, morphine, strychnine nitrate, quinine hydrochloride and quinine sulfate, quinidine sulfate, atebrin, caffeine, albucide, sodium norsulfazol and piperidine tartrate) was studied with the use of frog corneas. In other experiments we used erythrocytes, endothelium, and fibrocytes from the frog, and corneal epithelium of the mouse. The corneas and livers were carefully dissected out with sharp instruments and placed in Ringer's solution of the appropriate composition (for cold-blooded or warm-blooded animals). With the tissues of the frog, the work was carried out at a temperature of 16 to 19°C; containers with the mouse tissues were

placed in the oven at 37°C, and only at the time of preparation and microscopic examination were they at room temperature.

Ordinarily, preparation of the tissues was followed by a "rest" (20 to 30 minutes) for the isolated tissues in Ringer's solution, after which they were transferred into Ringer's solution containing novocaine or some other of the test agents. Microscopy was carried out after different periods of time, but usually 30 minutes after onset of exposure to the agent. Usually, after 30 minutes of exposure to the substance, ordinarily at high concentrations, the fragments of tissue being studied were transferred into pure Ringer's solution and then 30 minutes later (or at some other time), were again studied under the microscope in order to determine possible reversibility of the changes evoked.

Microscopy and documentation of the observations by drawings were carried out with the aid of a drawing apparatus with a magnification of about 600 times (water immersion x 40 with an aperture of 0.75 and ocular x 15). In a number of cases we applied vital staining to cornea or to parts of the liver, both of controls and of tissues subjected to the action of one or another agents, for 20 to 30 minutes in 0.02 percent solution of neutral red in Ringer's solution without soda. Prior to microscopy, the stained tissues were ordinarily placed for 20 to 30 additional minutes in Ringer's solution, according to the method of Aleksandrov (1949).

The Effect of Novocaine on the Cells of Isolated Tissues

Control frog corneas, not exposed to the action of pharmacologic agents, were studied after being kept for definite periods of time in Ringer's solution. For microscopy of the corneas, the latter were placed in a drop of Ringer's solution under a cover slip with the external epithelium uppermost. The latter, as is known, is a multilayered nonkeratinizing tissue. At places on the very surface of it, there are dead, flat cells which can be easily recognized by their sharp borders and their clearly-structured nuclei. Beneath these are live epithelial cells: one to two layers of flat epithelial cells, then two to three layers of prickle cells, and finally a basal layer of tall, prismatic cells.

The cytoplasm of the epithelium during the first two to three hours of incubation in Ringer's solution is very translucent, but later becomes slightly turbid. We could discover no structures or inclusions in it under our conditions of study even with prolonged retention (more than a day) in Ringer's solution, unless one includes fine, scarcely detectable, luminiscent granulations in the superficial

live cells. An exception are the cells injured by the dissection, in the ctyoplasm of which there may be a few inclusions which seem to be of a vacuolar character.

The nuclei in the cells of all layers are not invisible, with the exception of the superficial dead cells and those injured by dissection. With vital staining, numerous small granulas of neutral red are found in the liver superficial and in the prickle cells (Fig. 1a), while in the basal cells there are very few granules (Fig. 1b). The region about the nucleus does not contain granules. The cytoplasm and the nuclei remain practically unstained. About the edge of the section the cells stain diffusely at early stages of injury; however, after being placed in Ringer's solution, many of them regain their capacity to concentrate granules of the dye, the granules in this case being considerably larger and more numerous than in intact cells.

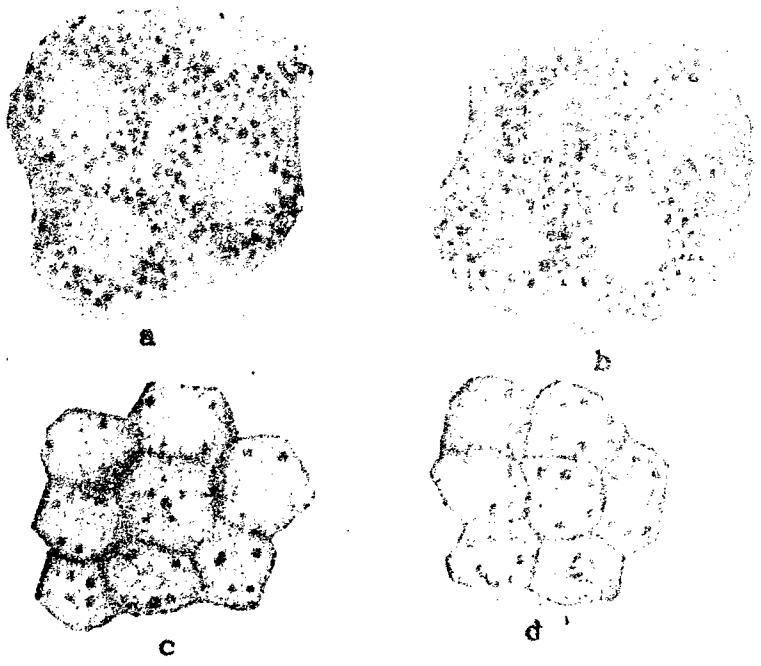

Fig. 1. The External Epithelium of the Frog Cornea.
a -- precipitation of granules of dye in the prickle cells (stained with 0.025 percent neutral red for 30 minutes); b -- same in basal cells; c -- precipitation of novocaine inclusions in prickle cells (exposure to an 0.25 percent solution of novocaine for 30 minutes); d -- same in the basal cells.

When we placed corneas in 0.25 to 0.5 percent solutions of novocaine, within ten to 15 minutes small round

inclusions appeared in the epithelial cells. Like the granules of dye, these were formed only in the cytoplasm, and were more numerous in the flat and prickle cells and less numerous in the basal cells (Figs. 1c and d). In the latter they may be distributed in groups in the apical zone of the cell, but more frequently are found in the edges, away from the nucleus, not infrequently forming what appear to be rings of beads. With increase in the time of exposure to novocaine to 30 to 60 minutes, the number of novocaine inclusions increased somewhat and their dimensions likewise increased.

Upon transferring these corneas into Ringer's solution, the number and size of the inclusions increased slightly, especially after preceding exposure to 0.5 to 1 percent solutions of novocaine, but there were no important changes during the first two to three hours of being kept in the pure solution.

During the early stages of development, the smaller novocaine inclusions conveyed the impression of granules, whereas the larger droplet inclusions looked more like vacuoles, since they were more translucent than the cytoplasm. Upon vital staining of cells with novocaine inclusions, neutral red was precipitated within these inclusions, which therefore took on the appearance of heavy granules of the dye. The nucleus and cytoplasm of the cells did not stain, which indicates the absence of any significant changes in the direction of paranecrosis. On the other hand, this was evidenced by other experiments with the use of unstained preparations, since the nuclei and nucleoli even in 0.25 to 0.5 percent solutions of novocaine remained unchanged for a period of one to one and a half hours, as a rule.

We studied the effects of different concentrations of novocaine. In the range of 0.25 to 1 percent, increases in the concentration caused a gradual increase in the rate of appearance of the inclusions and in the number of them during 30 minute periods of time to exposure to the agent. Although at the lowest concentrations, the formation of novocaine inclusions was delayed, nonetheless keeping the corneas for many hours in these solutions caused a marked filling of the cells with gradually enlarging novocaine inclusions.

The effects of high concentrations of novocaine (2 to 10 percent) will be described below. The epithelium of the frog cornea was used as a primary object of study. In the whole series of experiments in vitro, we were able to convince ourselves that similar novocaine inclusions were formed also in other cells -- in erythrocytes, fibrocytes, macrophages, in the epithelium of Descemet's membrane, the epith-

elium of the skin, and in the parasympathetic neurons of the frog, as well as in the corneal epithelium of the mouse. In all of these cases, the distribution of novocaine inclusions very closely duplicated the picture of distribution of granules of neutral red.

The Action of Novocaine on Cells in Situ

In order to determine whether there is formation of novocaine inclusions in cells in situ, we arranged experiments on intact frogs. Initially, the appearance of typical novocaine inclusions could be seen in the corneal epithelium following repeated applications of drops of 0.5 to 2 percent solutions of novocaine into the eyes of the frogs over a period of an hour. The other eye of the same frogs were used as controls -- pure Ringer's solution was applied in drops; no inclusions were seen in the epithelium of the control corneas.

In addition, we set up long-term experiments in which the frogs were kept for many days in weak (0.025 to 0.25 percent) solutions of novocaine in tap water. Under these conditions also, the characteristic novocaine inclusions appeared. The rate of their formation was proportional to the concentration of novocaine. Thus, in 0.25 to 0.1 percent solution, even on the second day of exposure to novocaine, abundant large novocaine inclusions appeared in the epithelium of the cornea and the skin of the natatory membrane, as well as in the erythrocytes. In 0.025 percent solution, after 40 hours of exposure to novocaine, there were no inclusions in the erythrocytes and only a few in the cornea and skin. After 65 hours there were a few more, and some appeared also in the erythrocytes. With longer periods of time in 0.025 to 0.05 percent solutions of novocaine, inclusions filled the epithelial cells of the cornea and skin, became much heavier, especially in the skin, and acquired a distinctly vacuolar character. In this stage, the cells are very reminiscent of those described by Kamnev (1933) as "stuffed" with large inclusions of neutral red, when speaking of cells of epithelium exposed to prolonged action of this dye. In erythrocytes, the small novocaine inclusions initially often fuse into a single large vacuole upon prolonged exposure to the agent. In the later stages, novocaine inclusions were found also in the endothelium of vessels. At concentrations of 0.01 percent novocaine in water, no inclusions were seen, even after two weeks of keeping the frogs in the solution.

Experiments on the effect of novocaine in vivo showed that even marked overdistention of the epithelial cells with large novocaine inclusions did not suppress their life acti-

vity. This is indicated by the fact that the nuclei of these cells remain invisible in the living preparation, and that upon staining with neutral red, only the novocaine inclusions take up the dye, whereas the cytoplasm between them and the nuclei practically does not stain at all. Moreover, in fresh preparations of frog skin fixed and stained in eosin-azure, after exposure for three to four days to 0.05 to 0.025 percent solutions of novocaine, one can detect mitoses in markedly vacuolated cells of the epidermis.

The Effects of Other Substances on the Isolated Frog Cornea

We wanted to determine how specific for novocaine is the formation of the unique colorless inclusions. In connection with this, we studied the effect on the frog cornea of a number of other pharmacologic agents.

It turned out that, in 0.01 to 0.25 percent solutions of strychnine, numerous inclusions are formed in the external epithelium and in Descemet's epithelium, as well as in the fibrocytes of the cornea and in the erythrocytes of the frog. These inclusions are altogether similar in appearance and distribution to novocaine inclusions and to granules of neutral red. In weaker concentrations of the agent, inclusions are precipitated more slowly (in 0.01 to 0.05 percent solutions, after one and a half to two hours). In 0.25 to 0.5 percent solutions, the inclusions are larger and arise within ten to 15 minutes. Their number increases after the corneas are kept in pure Ringer's solution.

Strychnine inclusions, like novocaine inclusions, selectively take up neutral red upon vital staining. This gives us reason to suggest that the increase in formation of granules of neutral red in the first phase of alteration of the epithelium, described by Levin (1951) after exposure to 0.2 to 0.3 percent solutions of strychnine, is explained not only by the more intensive precipitation of newly-formed granules of dye, but also by the staining of numerous large strychnine inclusions.

Inclusions which are indistinguishable in appearance from novocaine and strychnine inclusions appear in the epithelium and fibrocytes of the cornea following exposure to quinine, quinidine, and atebrin. Quinine and atebrin inclusions appear in large numbers in the epithelium within 30 to 60 minutes after exposure to 0.001 to 0.005 percent solutions of these substances. Hence, the threshold for the formation of inclusions is 5 to 10 times less for quinine and atebrin than for novocaine. An increase, within certain limits, of the time of exposure or of the concentration of these agents is accompanied, just as in the case of novocaine, by an increase in the number and size of the inclusions, upon

which they acquire a large vacuolar appearance.

With cocaine (0.1 to 0.5 percent) and morphine (0.1 to 0.5 percent) we ran only a few experiments, in which, in the cytoplasm of different cells of the cornea, we noted the formation of characteristic, colorless inclusions which were quite similar to the novocaine, strychnine, quinine, quinidine, and atebrin inclusions.

In addition, a number of the tested substances (0.1 to 0.5 percent caffeine, 0.2 to 2 percent albucide, 0.1 to 10 percent sodium norsulfazol, 0.1 to 1 percent piperidine) in these concentrations did not cause the formation of any inclusions in the cells.

These findings incidate that novocaine and a number of other alkaloids induce the appearance of similar granular and vacuolar inclusions in different cells which resemble in appearance the granules of vital dyes.

In the course of the experiments we also accumulated some facts which indicate that this similarity is apparently not superficial, but is due to a certain commonality of mechanisms of formation of inclusions of one or another type.

Remarks Concerning the Similarity of the Formation of Novocaine and Other Alkaloid Inclusions to the Inclusions of the Basic Granular Dyes

It has been shown (Yasvoin, 1925; Nasonov, 1926; Kryukova, 1929, and others) that granules of the basic vital dyes are precipitated in the area of the Golgi apparatus. The constant similarity of the localization in different cells of novocaine and other alkaloid inclusions to the localization of granules of neutral red compels us to believe that the alkaloid inclusions also are precipitated in the area of the reticular apparatus. However, in the cells with which we experimented (corneal epithelium, epithelium of the skin, fibrocytes, and erythrocytes) it was difficult to determine whether there was a connection between the inclusions and the reticular apparatus, since the elements of the latter are rather diffusely distributed in these cells.

A suitable study object for a solution of this problem is the liver cell, in which the elements of the apparatus are very regularly oriented about the edges of the cell along the bile capillaries (Nasonov, 1926, Makarov, 1926, 1931).

In our experiments the liver of a mouse was sectioned into small portions. One or two portions served in each experiment as a control and were studied without staining or after staining with 0.025 percent neutral red. Other portions were exposed to novocaine (0.2 to 0.5 percent). For

microscopy, we prepared fine sections from the control and from the experimental portions, with the aid of a sharp razor which we then placed in Ringer's solution under a cover slip.

Studies of the control material showed that the nuclei of undamaged liver cells are scarcely detectable, whereas fine mitochondria are visible in the cytoplasm. Sometimes the cells are found to contain different inclusions, particularly fatty (shining) inclusions. Mice in the livers of which there were many such irrelevant inclusions were excluded from the experiment.

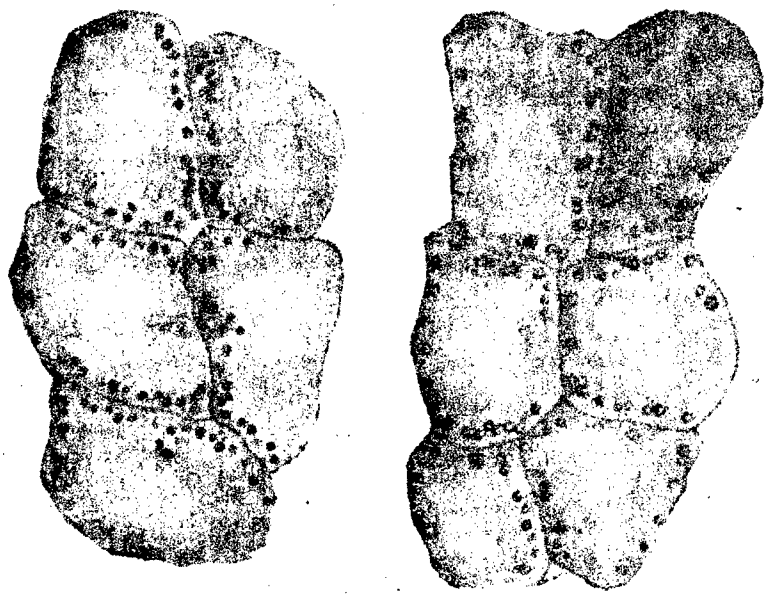

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Fig. 2. Liver Cells of the Mouse

a -- precipitation of granules of dye along the course of the bile capillaries (stained with 0.025 percent neutral red for 30 minutes); b -- precipitation of novocaine along the course of bile capillaries (effects of 0.2 percent novocaine over a 30 minute period).

In the cells of the mouse liver, granules of neutral red are precipitated primarily along the edges in the area of distribution of the reticular [Golgi] apparatus. (Fig. 2a). Exposure of pieces of liver to 0.2 to 0.5 percent solutions of novocaine in Ringer's solution causes the formation of turbid, slightly yellowish-rose inclusions very similar to granules of dye, which are also distributed primarily in the areas of localization of the Golgi apparatus (Fig. 2b). In weaker concentrations of novocaine, numer-

ous small inclusions were formed, whereas with higher concentrations (0.5 percent) the inclusions were larger and fewer.

On the basis of our experiments with liver cells there is reason to believe that the similarity of distribution of the granules of the dye and of novocaine inclusions in different cells is not accidental and depends on the formation of one or the other of these in the area of the reticular apparatus. This is a fundamental argument in favor of a definite analagy between novocaine and granules of dye.

A characteristic features of the granules of vital dyes is that they are precipitated only in intact or slightly damaged cells. With certain degrees of injury (paranecrosis) and also after death of the cells, granule deposition did not occur (Nasonov and Aleksandrov, 1930). In connection with this, considerable interest attaches to the deposition or precipitation of novocaine and other alkaloid inclusions in severely injured or dying cells.

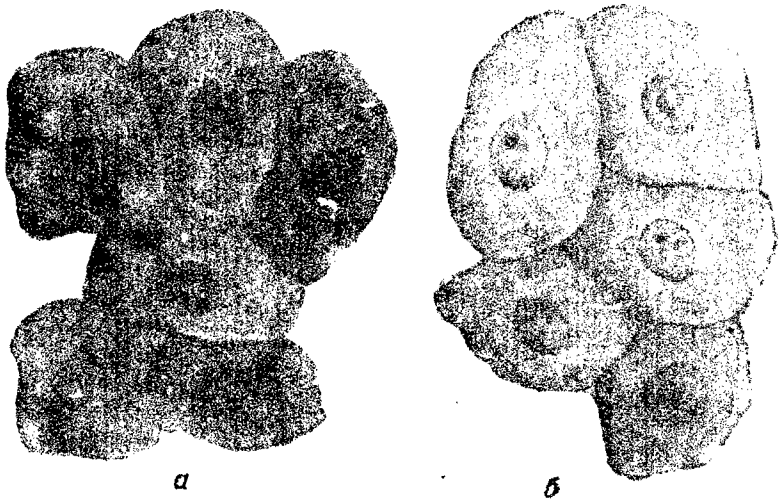

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Fig. 3. Liver Cells of the Mouse Heated for Five Minutes at 46°C.

a -- diffuse staining of nuclei and cytoplasm (stained with 0.025 percent neutral red for a period of 30 minutes); b -- absence of precipitation of novocaine inclusions (exposure to 0.2 percent novocaine).

As early as the first experiment on mouse liver, we noted that novocaine inclusions did not form along the region of the cut, where cells were noted to have clearly-structured nuclei and a turbid, gelatinized cytoplasm and,

upon staining, gave a picture of paranecrosis.

Later, in a number of experiments, we were able to show that, upon staining cells of the liver with neutral red after a five minute heating at 46°C, the dye is distributed diffusely in the cytoplasm and stains the nuclei of the cell (Fig. 3a). In accordance with this, in these injured cells there was a marked suppression, and even an absence, of the formation of novocaine inclusions (Fig. 3b).

A similar suppression of the formation in liver cells both of granules of neutral red and of novocaine inclusions can be achieved by diluting the Ringer's solution four times. Upon doing this, the liver cells swell up slightly, their cytoplasm becomes more granular and slightly turbid, and the nuclei acquire a very distinctive structure. With vital staining, both the cytoplasm and the nuclei stain diffusely.

It is also known that more concentrated solutions of granular dyes themselves suppress granule formation; hence, with increases in concentration, their toxic effects are intensified. In this respect, we also noticed a similarity in the behavior of granular dyes and novocaine.

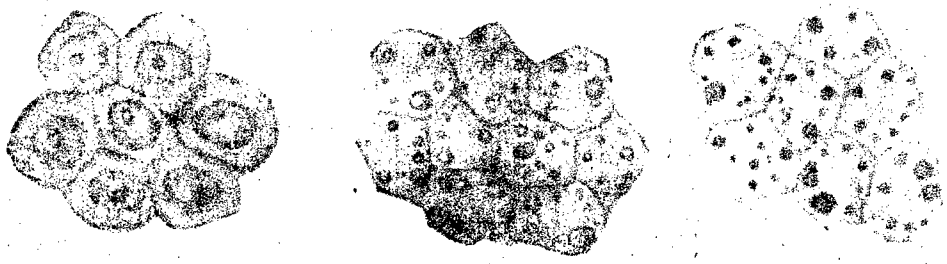

Fig. 4. The Basal Epithelium of the Frog Cornea

a -- 15 minute exposure to six percent novocaine; the characteristic novocaine inclusions are missing, and nuclei and nucleoli are visible; b -- same preparation after two hours of being kept in Ringer's solution; the nuclei again have ceased to be discernible, and numerous novocaine inclusions have formed; c -- the same preparation stained with 0.025 percent neutral red after "reversing"; the cytoplasm and nuclei do not stain, but dye accumulates in the novocaine inclusions.

It has been mentioned above that an increase in the concentration of vovocaine to one percent causes an intensification of precipitation of novocaine inclusions upon 30 minutes of exposure of the cornea to this concentration.

However, more concentrated solutions of novocaine, such as two percent and especially four to ten percent, quickly cause damage to the cells and, simultaneously, markedly suppress the formation of novocaine inclusions (Fig. 4a). Injury of cells is exhibited primarily in the appearance of hitherto indiscernible nuclei and nucleoli which, as is known, is one of the signs of paranecrosis. The ctyoplasm becomes slightly turbid. In a two percent solution of novocaine, these changes arise within 30 minutes. Prior to this time, a certain number of inclusions may be formed, which ordinarily have the shape of large vacuoles; however, their number and size do not further increase.

With increases in concentration of novocaine, the "appearance" of nuclei comes on more quickly: in fiveto six percent solution they are readily visible within lo to 15 minutes after onset of exposure to the anesthetic, and in 10 percent solution withi five to eight minutes. Accordingly, in 5 to 10 percent solution there is a more marked suppression of the formation of novocaine inclusions, which in many experiments were absent almost altogether. The effect of suppression is sometimes more marked in the deeper layers of the epithelium, especially in the basal layers.

Changes in the cells upon exposure to 5 to 10 percent solutions of novocaine are reversible if the exposure is not prolonged, that is, there is paranecrosis but not death of the cells. This is evidenced by the fact that, if corneas are kept for one or more hours in Ringer's solution after being exposed for 15 to 30 minutes to a four to six percent, or eight minute to 10 percent, solution of novocaine, the nuclei and nucleoli again become indiscernible, the cytoplasm becomes less turbid, and numerous novocaine inclusions are formed in it (Fig. 4b). These inclusions are frequently very large and have the form of droplets. Staining of these "rested" corneas indicates the absence of paranecrosis: the nuclei and cytoplasm do not stain, and the dye is concentrated in the novocaine inclusions (Fig. 4c).

In certain experiments it was possible to observe suppression of the formation of novocaine inclusions in solutions of high concentration. The same thing happened in mouse liver cells and in frog erythrocytes. In these cases, suppression was accompanied by the "appearance" of the nuclei.

Certain findings on the effects of high, toxic concentrations were obtained for a number of other alkaloids. Thus, a 30 to 50 minute exposure to 0.75 percent to 1 percent solutions of strychnine caused rounding up of epithelial cells and "appearance" of their nuclei, and considerably suppressed the formation of strychnine inclusions. In 0.5 per-

cent solution, this occurred after one and a half to two hours and was less pronounced.

An inhibitory influence of sufficiently high concentrations on the formation of inclusions was observed also in the case of morphine (one percent) and quinidine (0.1 percent). Timely transfer of corneas exposed to these agents into pure Ringer's solution was likewise accompanied by disappearance of visible structurization of nuclei and by the development of numerous inclusions.

The localization of novocaine inclusions in the area of the Golgi apparatus and the reversible suppression of their formation upon profound injury of cells strengthened our initial impression of the similarity of the behavior in cells of novocaine (and apparently a number of other alkaloids) to the behavior of the basic granular dyes. However, because of the absence of color of these agents, we were unable to demonstrate whether they accumulated within the inclusions as did the granular dyes. For a solution of this problem we took advantage of the natural fluorescence of quinine, quinidine, and atebrin. The distribution of these agents in the cell was studied with the aid of fluorescent microscopy, using the system of Ye. M. Brumberg. The microscope MBI-1 was fitted with a lens OI-17 and with an illuminating source OI-18, or with a more powerful source of light -- the SVDSH-250 bulb. Observations were carried out using incident light, either in short blue rays (filter FF_1 with a maximum filtration in the area of 400 millimicrons) or in long untraviolet rays (filter UFS_3 with a maximum filtration in the area of 360 millimicrons). In working with the filter FF_1 we used a protective filter P-2N, and when using the filter UFS_3, we used a protective filter ZhF_{18}.

In studying quinine, quinidine, and atebrin inclusions under fluorescent microscopy, we discovered a selective fluorescence of inclusions, whereas the cytoplasm and nuclei were practically devoid of luminescence. Fluorescence of the inclusions corresponds in color and intensity with the fluorescence of solutions of the respective agents in Ringer's solution. Thus, a very sharp fluorescence of a yellowish-green hue is characteristic both for solutions of atebrin and for "atebrin" inclusions, in studies carried out with the FF_1 filter and the protective filter P-2N. In studies with the UFS_3 filter and the protective filter ZhS_{18}, fluorescence in both instances has a bluish-green color.

Quinine and quinidine inclusions fluoresce less brightly and, hence, it is expedient to use a more powerful light source -- the SVESH -- 250 bulb. Under these conditions, the quinine inclusions give a bright blue luminescence with the UFS_3 filter; a similar fluorescence was detected in solutions of

similarity in the morphology of granular and vacuolar inclusions formed upon exposure to novocaine, quinine, and certain other alkaloids to the granules of dye; (2) the development of each of these in the region of the Golgi apparatus; (3) suppression of precipitation both of "alkaloid" inclusions and of granules of dye following a certain degree of injury to the cells; (4) a selective accumulation of quinine, quinidine, and atebrin in the inclusions.

At first glance, a similarity in the behavior within the cell of such different substances as novocaine and neutral red may seem highly improbable. However, Nasonov (1923, 1926, 1928), Yasvoin (1925), Veyner (1928), Makarov (1931) and others have shown that various substances -- water, secretion, fat, certain basic dyes, and iron salts -- may be incorporated in the cytoplasm in the form of inclusions. It is noteworthy that in all these cases the inclusions are formed in the region of the Golgi apparatus which is, according to Nasonov, the secretory organoid of the cell. According to Fel'dman (1947), the incorporation of various substances in the form of inclusions in the region of the Golgi apparatus is based upon a common coacervation mechanism; with respect to the dyes, he has been able to demonstrate this in model experiments. It is of interest that novocaine, strychnine, and quinine, in the model experiments of Bungenberg de Iong (cited by Fel'dman, 1947), also formed coacervates.

It is therefore possible that the alkaloid inclusions which we studied, just like the granules of dyes, are coacervates within the cytoplasm.

Of course, the present work does not give us reason to state that the inclusions which we studied are altogether comparable to the granules of dye in significance or with respect to the mechanism of their formation. Thus, for example, the accumulation of alkaloids in the inclusions has been demonstrated only in the case of the fluorescing agents. The absence of typical substrate in the "old" novocaine inclusions testifies to the possibility of a number of differences in specific processes occurring upon the formation of alkaloid inclusions and of granules of dye.

Nonetheless, the above mentioned facts give us reason to assume the possibility that there is precipitation of alkaloids in the region of the Golgi apparatus. In any case, such an interpretation may now be adopted as a working hypothesis for further analysis of the distribution of different pharmacologic agents within cells.

Conclusions

1. Novocaine, cocaine, strychnine, morphine, quinine, quinidine, and atebrin, in definite ranges of concentration, induce the formation of colorless inclusions in the cytoplasm.

2. These inclusions are superficially reminiscent of the granules of vital dyes and show a number of similar traits: they also are formed in the region of the Golgi apparatus, their deposition is inhibited by influences which cause profound paranecrosis or death of the cells. For fluorescing agents (quinine, quinidine, atebrin), we were also able to demonstrate the accumulation of these agents within the inclusions.

3. The significance of this process may consist in the intracellular elimination of harmful agents. This proposition is in agreement with ideas concerning the protective importance for the cell of granular precipitation of dyes and with ideas concerning the secretory functions of the Golgi apparatus.

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Intravital Changes of Nerve Cells in the Intact Organism Under the Influence of Novocain

M. V. Makhover

The Department of General Biology of the Leningrad Sanitation and Hygiene Medical Institute

The present work involved studies utilizing the method of intravital staining for the detection of changes occurring under the influence of novocain in nerve cells and other animal cells. Intravital staining, as has been shown by a number of studies (See Nasonov and Aleksandrov, 1940; Nasonov, 1959), affords us the possibility of appraising changes in the protoplasm which are not detectable by the methods of cytologic analysis. Intravital staining may be used not only for isolated organs and tissues but also for the entire organism.

Methods

The studies were carried out on male autumn-winter frogs and on white mice. Intravital changes under the influence of novocain were studied in cells of the sympathetic nerve chain, liver, and kidneys, and, in frogs, in the parasympathetic cells of the heart, as well.

Prior to the experiment, the animals were weighed and then injected with a one percent solution of neutral red, calculated for a dosage of 0.3 to 0.5 mg of dry dye per gram of body weight of the animals (for mice this was 0.3 milligrams). Control animals were given only the dye, experimental animals were preliminarily injected with novocain, either into the body cavity or into the lymph sac. Experiments to determine the effect of each dose were set up on five to ten animals. An hour and a half after the injection of novocain, the animals were autopsied and the organs of interest removed from them. Isolation of the heart was effectuated by the method of M. I. Grimenitskiy (1939). For studies of such organs as the liver and kidney, small sections were taken from them which were then placed in a drop of Ringer's solution, covered with a cover slip, and viewed under the microscope. Part of the animals were kept as controls for determination of reversibility and for studies of the ensuing changes in the distribution of the dye.

Experiments on Frogs

Control. After the administration of one percent solu-

tion of neutral red, a normal picture of intravital staining was observed in the nerve cells being studied (Fig. 1a; 2a). In the cytoplasm there appeared a large number of uniformly distributed orange-red granules of dye which surrounded the area of the colorless nucleus. The nucleolus in the majority of cases could not be detected. The injection of the dye showed no effects on the behavior of the animals.

Twenty to 30 minutes after the injection of neutral red, weak staining of the cytoplasm could be detected in the cells of the sympathetic nerve chain, and a more distinct staining in the cells of the spinal ganglia). The area of the nucleus could not be made out. Beginning 40 to 50 minutes after the injection of neutral red, there first appeared very fine, then larger granules. By 80 to 90 minutes, the area of the colorless nucleus was clearly defined.

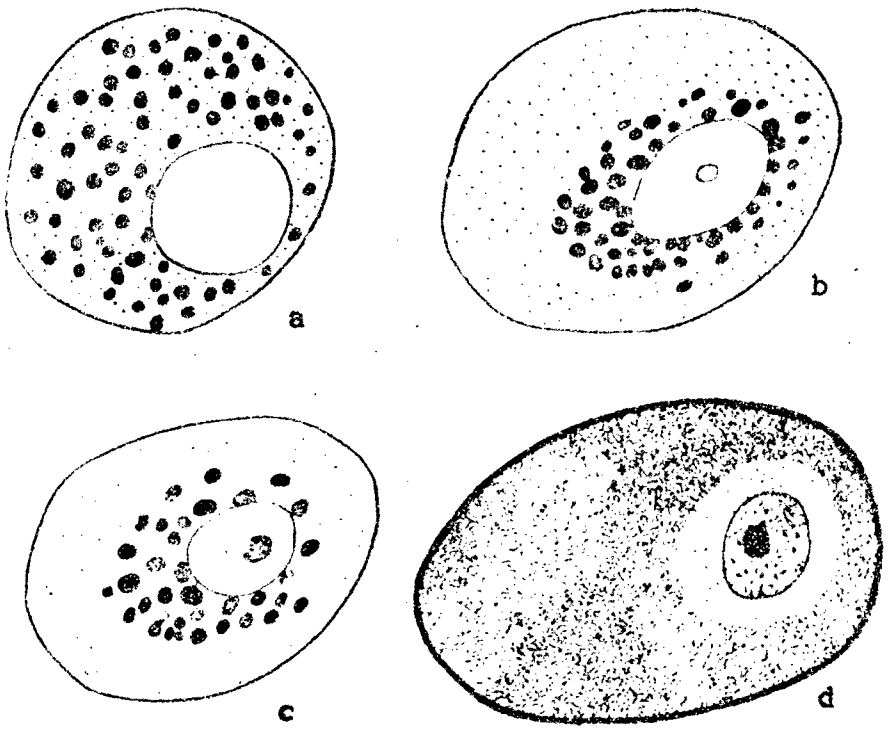

Fig. 1. Cells of the Sympathetic Nerve Chain of the Frog (intravital staining with neutral red).

a -- control, b -- minimum paranecrotic dose of novocain, c -- minimum lethal dose of novocain, d -- lethal dose of novocain.

After this, autopsy of frogs was carried out regularly every twenty-four hours. The last group of frogs was autopsied 10 days after injection of the dye. In the hep-

atic cells and in the cells of the renal tubules, clearly defined granules could be seen throughout the course of the entire experiment. The brain, which initially stained with an orange-red hue, with granules concentrated, in definite areas of the cells, acquired a raspberry color by 48 hours in a number of cases. By the eighth to tenth day the cerebral cortex was stained a weakly orange-red hue. Microscopic examination of the cerebral hemispheres showed rather numerous orange-red granules. In the spinal cord, the staining of which was considerably less intense than that of the brain, clear granules could be seen by the seventh to tenth day. In the motor cells, these granules were not large and were concentrated primarily at the periphery of the cells.

In the cells of the sympathetic nerve chain, granules could be seen for as long as six days. By the seventh to tenth day, distinctive granules could no longer be seen in some cells and the staining was a raspberry color. In the spinal ganglia, this picture, at the same date, was seen primarily in the small cells. In the larger cells the granules were still clearly visible. It should be noted that, in some cells, faintly staining nucleoli could be discerned in colorless nuclei.

From what has been said it follows that, in cold-blooded animals during life, the method of intravital staining may be used both in acute and in long-term experiments.

Morphological Changes in Nerve Cells Under the Influence of novocain. We studied the changes of intravital staining in the presence of minimum narcotic, minimum paranecrotic, and minimum lethal doses of novocain. As the minimum narcotic dose we used a dose which would cause a state of narcosis lasting from 20 to 40 or 45 minutes. A paranecrotic dose was considered one which would lead to deviation from the normal picture of intravital staining. Finally, a minimum lethal dose was taken to be a dose which, in the presence of a prolonged narcotic state, was accompanied by more marked disturbance of the cells and would lead to death in about 50 percent of the experimental animals.

The experiments showed that novocain, beginning with 0.17 milligrams per gram of body weight, causes general narcosis of frogs; this dose, therefore, was used as the minimum narcotic dose. Forty to 50 minutes after the injection of novocain, we observed restoration of functions in frogs, with the corneal reflex being the first to be restored. Microscopic studies of nerve cells showed that minimum narcotic doses did not cause any changes in them. The injection of 0.33 mg. to 0.66 mg. of novocain per gram of body weight of the animals produced similar results.

Doses of novocain equal to 0.9 and 1 mg. per gram of

body weight of the animals may be considered minimum paranecrotic doses. Upon exposure to these doses, the cytoplasm of the cells of the sympathetic nerve chain stains a pale rose color, and there are two or three rows of red-orange granules surrounding a colorless nucleus and a clearly visible nucleolus (Fig. 1b).

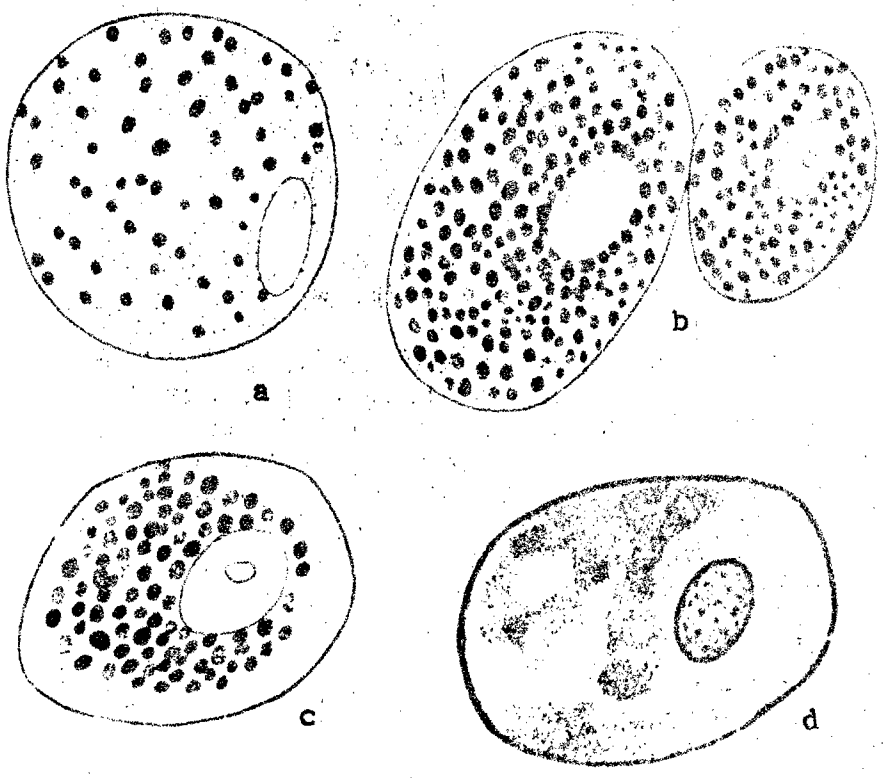

Fig. 2. Sensory Cells of the Spinal Ganglia of Frogs (intravital staining with neutral red).

Designations the same as in Fig. 1.

In the cells of the spinal ganglia under the same conditions, we detected intense granule formation. The area of distribution of the nucleus appeared as a colorless spot. In the smaller cells of the ganglia, the contours of the nucleus and nucleolus were more distinct (Fig. 2b). As to the parasympathetic cells of the heart, deviations from normal of these cells appeared only in the fact that the colorless nucleus and nucleolus were more distinct.

A dose of novocain of 1.3 mg. per gram of body weight of the animal proved to be a minimum lethal dose. An hour and a half after the injection of this solution, and also upon raising the dose to 1.6 mg. (the latter causes death in

frogs), granule formation in all types of cells studied was not completely suppressed. In the cells of the sympathetic chain, a very small number of granules of the dye formed a corona around the faintly stained nucleus containing an easily-distinguishable nucleolus (Fig. 1c). In the sensory cells of the spinal ganglia, large numbers of heavy granules were concentrated in the central portion of the cell. The nucleus, nucleolus, and cytoplasm were faintly stained (Fig. 2c). In the parasympathetic cells of the heart there was a slight diminution in the number of the granules. The nucleus, nucleolus, and cytoplasm were only faintly stained. The neutral red took on a raspberry hue, which testifies to the shift of the reaction to the acid side.

With lethal doses of novocain (3.3 mg. per gram body weight of the animal), the color of the cells of the sympathetic chain was a brick red, and the nucleus, which was surrounded by a non-staining zone, was clearly structured (Fig. 1d).

The nucleus was bright red. In the spinal ganglia the color of the cells was brick red, but less intense than in the chain. Granules were absent, the nucleus was structured, and the nucleolus could not be seen (Fig. 2d).

The absence of deviations from normal in the nature of the intravital staining in the presence of minimum narcotic concentrations of novocaine confirms the idea that narcosis of highly developed organisms is not accompanied by morphologic changes in the nerve cells (Makarov, 1938).

It may also be regarded as established that the different types of cells which we studied react differently to novocain. The most sensitive are cells of the sympathetic chain, then the cells of the spinal ganglia, and finally the parasympathetic cells of the heart.

Experiments on White Mice

Control. It should be remarked that some of the samples of neutral red proved to be toxic for white mice. In the case of non-toxic dyes, the mice readily tolerated the injection of a saturated solution (about two percent).

In this series of experiments we also determined how long the neutral red is retained in the organism of the mouse. On the basis of our observations it may be stated that, an hour and a half after injection of the dye, the organs may be arranged in the following sequence with respect to the intensity of their staining: liver > kidney > spinal ganglion > > cells of the sympathetic nerve chain > brain > spinal cord. After 24 hours, dye is still present in the liver and kidneys, but there is a slight diminution in the staining of cells of

the ganglia, and the spinal cord is colorless. A reddish-yellow tint is retained in some parts of the brain. Forty-eight hours after injection of the dye the liver is still intensely stained, the cells of the kidneys are much less intensely stained; granules of neutral red are seen only in a few isolated cells in the spinal ganglia, and in none of the cells of the brain or spinal cord.

Morphologic Changes of Nerve Cells Under the Influence of Novocain. Even in the first experiments we discovered differences in the reaction to novocain between warm-blooded and cold-blooded animals. Novocain in mice either showed no effect at all or else caused death in the animals within two to three minutes.

After prolonged attempts to make up a solution of novocain which would not cause death in the animals, we determined the following doses: minimum lethal dose (0.1 mg. of novocain per gram body weight of the animals), minimum paranecrotic dose (0.066 mg. per gram of body weight), and what we termed a sub-threshold dose (0.05 mg. per gram of body weight of the animal). With a minimum lethal dose, about half of the mice used in the experiments died within four to six minutes. In the surviving animals the acute manifestations gradually subsided, and within two hours they began to move about.

The minimum paranecrotic dose caused a restless stage in the animals. Gradually the mice quieted down and by the end of the experiment the majority of the animals were behaving just like the controls.

As the sub-threshold dose we used the maximum dose of novocain which caused no readily discernible changes in the behavior of the mice.

Microscopic studies showed different reactions in different parts of the nervous system to the action of the novocain. In the cells of the spinal ganglia, upon administration of sub-threshold dose, the cytoplasm and nuclei remained unstained. The dye, just as in controls (Fig. 3a), was deposited in the form of yellowish-red granules. Changes began to appear with the use of a minimum paranecrotic dose (0.066 mg. per gram body weight). In some experiments, in the presence of granules, the cells acquired a raspberry hue, with weak staining of the cytoplasm and nucleus, and in almost all cells there was intensified granula formation. Large numbers of heavy granules stood out clearly against the pale raspberry background of the cytoplasm. The area of the structureless nucleus remained unstained but the nucleoli were stained in a number of cells (Fig. 2b).

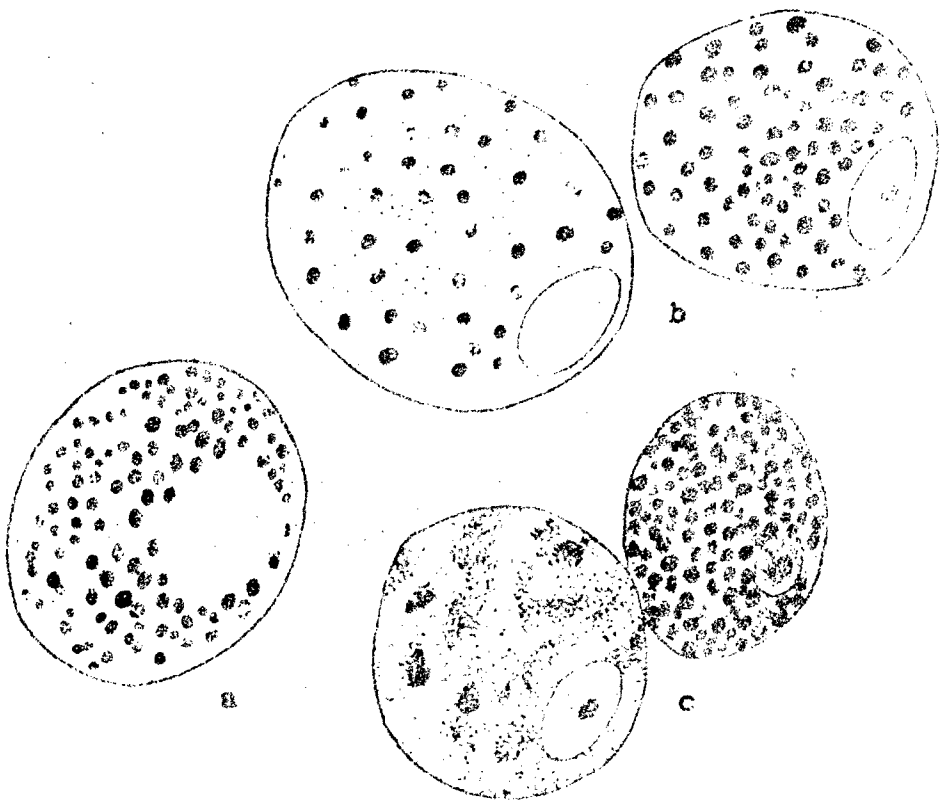

Fig. 3. Sensory Cells of the Spinal Ganglia of the White Mouse (intravital staining with neutral red).

Designations same as in Figure 1.

Upon injection of minimum lethal doses, in the majority of the experiments we noted an intensified granule formation. Granules of different sizes filled the entire cytoplasm. The sharply demarcated nucleus was unstructured, and the nucleolus could be seen in almost all cells. In some experiments we noted cells with diffuse staining of the cytoplasm, against the yellowish-red or raspberry background of which were concentrated large accumulations of dye in various places. The region of the nucleus was stained, but there was no structuring of the nucleus. The contours of the nucleus were distinct (Fig. 3c).

In the cells of the sympathetic nerve chain, changes under the influence of novocain were much less pronounced. With minimum toxic doses the region of the nucleus stood out much more clearly and the nucleolus could be seen. A raspberry hue of the cytoplasm was noticed only in a few isolated cells (Fig. 4b).

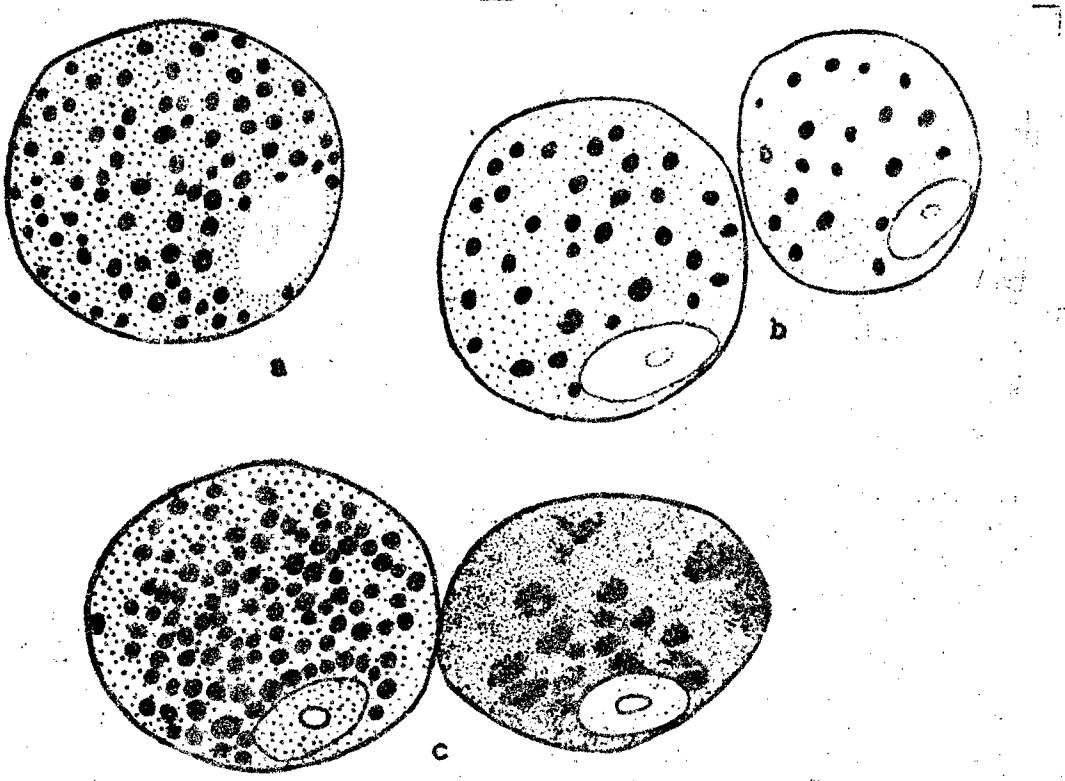

Fig. 4. Cells of the Sympathetic Nerve Chain of the White Mouse (intravital staining with neutral red). Designations same as in Fig. 1.

A minimum lethal dose caused in some cases an intensified granule formation; in others the granules were very few, while the cytoplasm stained a raspberry hue. The nucleus was unstained and structureless (Fig. 4c). Animals which remained alive and which were autopsied after 24 hours showed a normal picture (Fig. 4a). As to the brain and spinal cord, microscopic studies showed changes, at the concentrations which we used, only in the tone of the staining. The cells of the cerebral hemispheres acquired a raspberry hue with the granules being concentrated in the peripheral parts of the cells. In the spinal cord, the gray substance stained less intensely than did the brain, and the white substance was either unstained or stained a pale rose color. These findings indicate that novocain in certain concentrations causes a number of changes in the sensory cells of the spinal ganglia and in the cells of the sympathetic nerve chain, with changes in the cells of the spinal ganglia being the most pronounced.

With increases in the concentration of novocain, cells gradually undergo a transformation into a state of

stimulation, which it is possible to detect by the intensified granule formation. Later this state is superseded by suppression of vital functions, which is manifested in suppression of granule formation and also in staining of the nuclei and cytoplasm. All of the above described phenomena are reversible.

Conclusions

1. The method of intravital staining with neutral red under conditions in which the animals are kept alive may be used for cold-blooded (frogs) and warm-blooded (white mice) animals both under chronic and under acute experimental conditions.

The dye remains in the tissues of frogs for eight to ten days, and in white mice up to 24 hours, in the cells of the nervous system, and up to 48 hours and more in other tissues (liver, kidneys).

2. Novocain injected into the body cavity of frogs in certain dilutions causes a state of general narcosis. In white mice novocain, following intraperitoneal injection in effective doses, causes either stimulation or death of the animal. There is no narcotic stage.

3. The minimum narcotic dose for frogs (0.7 mg. novocain per gram of body weight) and the sub-threshold dose for white mice (0.05 mg. per gram body weight), do not cause any changes in the character of the intravital staining of the brain and spinal cord, the cells of the sympathetic nerve chain, the sensory cells of the spinal ganglia, and the parasympathetic cells of the heart.

4. With minimum toxic doses (0.9 to 1 mg. novocain for frogs and 0.066 mg. for white mice), and minimum lethal doses (1.33 mg. for frogs and 0.1 mg. per gram body weight for white mice), there are deviations from the normal which become evident in an intensification of granule formation, this being especially clearly exhibited in the sensory cells of the spinal ganglia.

5. The findings which we obtained indicate the stability of the cytoplasm of nerve cells and the cells of other tissues in the face of exposure to comparatively high doses of novocain, and likewise indicate the absence at these doses of profound or irreversible structural changes.

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The Absorption Properties of the Sartorius Muscle of Frogs Upon Increasing its Stimulability

I. P. Suzdal'skaya

Laboratory of Cellular Physiology of the Physiological Institute of Leningrad University

The idea that waves of spreading stimulation are based on processes similar to the paranecrotic reaction has been expressed in the monograph of D. N. Nasonov and V. Ya. Aleksandrov (1940), and has been confirmed and developed in the numerous works of D. N. Nasonov and his associates in the past ten years. It has been shown not only that parabiosis of nerve and muscle tissues caused by the effects of various modifying agents is accompanied by denaturative changes of the protein substrate, but also that the normal stimulation of muscle, nerves, and epithelial tissues caused by adequate physiologic stimuli is likewise characterized by an increase in absorption properties with respect to intravital dyes, which indicates changes in the proteins of the cytoplasm which are very similar in their nature to the denaturation of native proteins (Kiro, 1948; Romanov, 1948, 1953, 1957a, b, Vereschagin, 1949; Ushakov, 1950; Levin, 1952; Zarakovskiy and Levin, 1953; Nasonov and Suzdal'skaya, 1953, 1957; Chetverikov, 1953; Shapiro, 1953, Golovina, 1955a, Nasonov and Radvonik, 1956, and others). Intensification of absorption properties characterizes the later phase of the parabiotic reaction when the tissue is in a state of complete or partial refractoriness. Reduction in tinctorial properties has been described by many authors following exposure to weak subthreshold doses of modifying agents or upon emergence of tissues from a state of parabiosis (Romanov, 1949a, b, 1950; Golovina, 1955b; Kusakina, 1957, and others). In a number of works it has been pointed out that the phase of reduction in staining properties corresponds to an increase in the resistance of the tissues (Romanov, 1949, and others). However, no comparison of the level of stimulability of tissues with their absorption properties was made in these studies. Parallel studies of stimulability and of absorption by muscles of neutral red were made only in the studies of D. Ushakov (1951, 1954). According to his findings, the stimulability of the sartorious muscle of the frog under the influence of 0.15 percent potassium chloride increases, while the absorption of dye decreases as compared with the normal, whereas 4.5 percent ethyl alcohol and 0.25 percent chloral hydrate reduced both stimulability and staining properties. V. P. Proshina (1957) discovered that isolated muscles kept at a

temperature near 0°C undergo changes in their absorption capacities which correspond to phasic changes in stimulability. In the course of this, increased stimulability corresponds to a reduction in the absorption properties, and vice versa.

Hence, it seems that in some cases a weakening of absorption properties accompanies an increase in the stimulability of muscles, whereas in others, on the contrary, a reduction in stimulability is seen in these cases. Such an inconsistency in the experimental facts has not yet been satisfactorily explained.

The task of the present work is to make a more detailed study of the changes of the absorption properties of muscles upon increase in their stimulability.

Methods

Experiments were carried out on the sartorius muscles of frogs in the autumn and winter seasons. For staining we used phenol red prepared in Ringer's solution at a concentration of 0.03 percent, and neutral red (in our work we used neutral red produced by theChemical Plant imeni Voykov) in Ringer's solution without soda at concentrations of 0.01, 0.025, and 0.05 percent. The prepared muscles were kept in Ringer's solution for two to two and a half hours prior to the experiment until equilibrium was reached. Then both control and experimental muscles were simultaneously transferred into the dye solution. After staining, the muscles were sectioned in Ringer's solution and examined under the microscope. Muscles with injured fibers and also muscles undergoing contractions were excluded from the experiment. From the muscles, we sectioned portions which were equal in size and placed them for extraction of the dye in a measured amount of acidified ethyl alcohol. The alcohol extracts of the dye were studied photometrically in the Pul'frikh graduated photometer (phenol red) or in the electrophotocolorimeter (neutral red). The amount of dye absorbed by the object was expressed in extinction units times 100 and calculated for one gram of dry weight of muscle, or was expressed in percentage of the control value taken as 100 percent.

In cases in which it was necessary to stain dead muscles for comparison the muscles after preparation were killed by immersion for one hour in 48 percent ethyl alcohol (in Ringer's solution), after which they were washed in running water for a day. To determine complete saturation of the tissue with the dye, staining was continued for 35 hours. For characterization of the stimulability of the muscles, we determined either their voltage-duration curves or the A and B constants of the Gorveg formula, as was done in the work of

D. N. Nasonov and D. L. Rozental' (1953). For stimulation, we used a device with a capacity of 600 down to 0.01 micropharads. The long stimulability, or the reheobase (B) was determined in volts, and the short stimulability (A) in millivolts - milliseconds. The constants of stimulability were determined prior to immersion of the muscles in the dye and again upon termination of the staining period.

For evaluation of the reliability of the results, we used the Student-Fisher method of statistical processing. For confidence intervals, we used results in which the reliability (the value of alpha) was not less than 0.950.

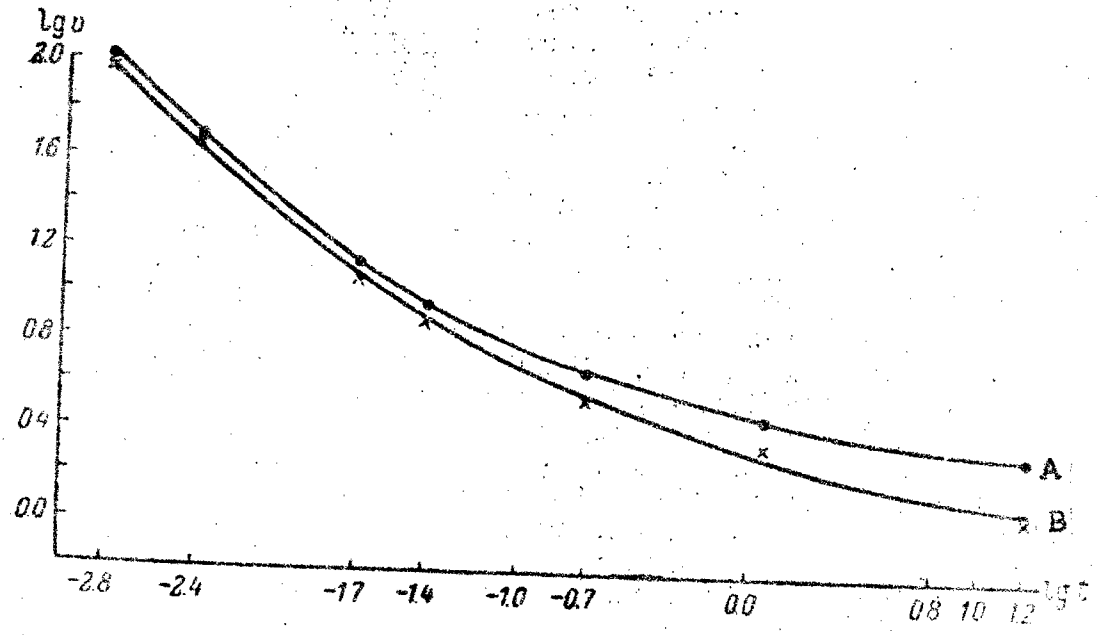
Results

We were interested in staining the cytoplasm at the moment of increased stimulability. It is known that the stimulability of conducting tissues is markedly increased by Ringer's solution which does not contain calcium chloride. In order to determine in what respect there is a change in the stimulability of the sartorius muscles under these conditions, we computed logarithmic-duration curves for control and experimental muscles. In the Figure, curve A (control) is based on measurements made after keeping the muscles for a period of 20 minutes in Ringer's solution containing neutral red in a concentration of 0.025 percent, curve B (experimental) was obtained from measurements of stimulability of muscles after being in Ringer's solution without calcium chloride with the same concentration of neutral red for 20 minutes. The points of the curves shown in the figure are average arithmetical results obtained in ten experiments. Table 1 gives the changes in stimulability of experimental and control muscles in percentages of the original level taken as 100 percent upon testing with currents of different duration. Thus, the stimulability of experimental muscles to a rheobase current of 24 microseconds duration increases by 46.5 percent; to a current of 0.2 microseconds duration, it increases by 20.6 percent and, finally, with short currents of 0.001 microseconds duration, the changes in stimulability are negligible. This material indicates that the stimulability of muscles kept in Ringer's solution without calcium is greatly intensified with respect to stimuli of rheobase duration. With shortening of the stimuli, the intensification of stimulability becomes progressively less pronounced.

Table 1

Changes in the Thresholds of Stimulability (in percentages of the original level) Upon Testing with Currents of Different Duration in Control and Experimental Muscles (M-arithmetic average, m-mean square error)

Duration of the current in milliseconds	Number of experiments	Control muscles (Ringer's + Neutral red 0.025 percent) M ± m	Experimental muscles (Ringer's without calcium chloride + neutral red 0.025 percent) M ± m
24.0	10	+ 9 ± 9.9	- 46.5 ± 4.0
0.2	10	+ 19.7 ± 17.8	- 20.6 ± 3.3
0.001	10	+ 6.2 ± 10.2	- 10.8 ± 4.9



Voltage-Duration Curves of the Sartorius Muscle of the Frog

A -- after the muscles were kept in Ringer's solution for 20 minutes; B -- after muscles were kept in Ringer's solution without calcium chloride for 20 minutes. Along the abscissa are the logarithms of time (in milliseconds); along the ordinate are the logarithms of threshold voltage (in volts).

The great intensification of stimulability of muscles to a rheobase current caused by the absence of calcium chloride in Ringer's solution is much to be preferred to the me-

thods of increasing stimulability which use special chemical agents, because of the possible adsorption competition between the modifying agent and the dye (Nasonov, and Aleksandrov, 1937). Nonetheless, in order to be completely certain that the absence of calcium chloride in Ringer's solution does not influence the staining process, we set up control experiments with staining of filter paper and of dead muscles according to a method developed by D. N. Nasonov and V. Ya. Aleksandrov, 1937, 1940). In this it was shown that absorption of the dye by filter paper and by dead muscles in solutions containing calcium and not containing calcium undergoes almost negligible differences, and the difference actually lies completely within the limits of experimental error. Consequently, the absence in Ringer's solution of calcium chloride does not influence to any noticeable extent the amount of the dye absorbed by the substrate.

Furthermore, it was necessary to determine whether the swelling of muscles upon staining them with neutral red in Ringer's solution containing calcium chloride and in Ringer's solution not containing calcium chloride was at all different. Different degrees of swelling of control and experimental muscles might strongly influence the amount of dye penetrating into the muscle, and hence might lead to erroneous conclusions concerning changes in the absorption properties of the muscular tissue. These experiments showed that the differences in the weights of prepared muscles stained under different conditions were negligible. On the average (of 10 experiments) the increase in weight of muscles kept for 20 minutes in a solution of dye not containing calcium chloride was 1.04 ± 0.92 milligrams. The increase in weight of muscles stained in normal Ringer's solution was 0.88 ± 0.70 milligrams. Hence, it was established that differences in swelling upon staining the muscles under these conditions could be disregarded.

Experiments on living sartorius muscles of frogs were carried out during the autumn and winter seasons. In all, we ran five series of experiments.

In order to determine the error of the method, we ran 22 experiments. Sartorius muscles were stained for 20 minutes with 0.025 percent neutral red prepared in Ringer's solution. Table II (series 1) shows the thresholds of stimulability of paired muscles in percentages of the original, and the absorption of the dye in units. From these figures it follows, first, that no statistically reliable changes occur in stimulability of muscles kept for 20 minutes in normal Ringer's solution in the presence of neutral red. Second, that the stimulability and absorption properties of paired muscles after staining in Ringer's solution show no statistically reliable differences.

Table 2

Stimulability and Absorption Properties of Sartorius Muscles of Frogs under Different Experimental Conditions. (M -- arithmetic average, m -- mean squared error, alpha -- reliability of difference)

Series of experiments	Conditions of experiments	Time of staining in minutes	Number of experiments	Changes in the threshold of stimulability in percentages of the original level				Absorption of dye $\frac{\text{Ext.}}{\text{Wt.}} \times 100$ (M ± m)	Alpha	Changes in absorption of dye (in percentage of control) $(\frac{O}{K} \times 100 - 100\%$
				Constant b (M ± m)	alpha	Constant a (M ± m)	alpha			
1	Ringer's solution + neutral red 0,025% (control)	20	22	— 0.6 ± 5.4	0.691	+24.2 ± 8.7	0.725	4.27 ± 0.24	0.617	+ 2.1
	Ringer's solution + neutral red 0,025% (experimental)	20	22	— 3.0 ± 3.9		+34.5 ± 14.8		4.36 ± 0.26		
2	Ringer's solution + neutral red 0,025% (control)	20	30	—12.2 ± 3.3	0.999	+ 8.9 ± 7.5	0.999	3.34 ± 0.18	0.998	+22.8
	Ringer's solution without calcium chloride + neutral red 0,025% (experimental)	20	30	—55.3 ± 3.3		—27.2 ± 3.2		2.58 ± 0.19		
3	Ringer's solution + phenol red 0,03% (control)	20	10	+21.7 ± 6.1	0.509	+18.2 ± 8.1	0.858	1.77 ± 0.09	0.539	+ 6.0
	Ringer's solution + phenol red 0,03% (experimental)	20	10	+22.7 ± 9.8		+ 5.0 ± 8.9		1.87 ± 0.07		
4	Ringer's solution + phenol red 0,03% (control)	20	37	+20.8 ± 4.9	0.999	+10.0 ± 4.3	0.993	2.93 ± 0.20	0.815	— 9.9
	Ringer's solution without calcium chloride + phenol red 0,03% (experimental)	20	37	—61.0 ± 2.2		— 6.3 ± 4.9		2.64 ± 0.19 (With a correction factor of 2,30)*		(With a correction factor of 21.5)*
5	Ringer's solution + 4.5% ethyl alcohol for 10 min. Ringer's solution for 10min. Ringer's solution + neutral red 0,05% (control)	10	20	+ 1.2 ± 2.9	0.999	+17.0 ± 6.9	0.945	4.90 ± 0.24	0.987	—16.3
	Ringer's solution + neutral red 0,05% (experimental)	10	20	—36.0 ± 3.1		+ 3.9 ± 5.1		4.10 ± 0.20		

*Note -- The correction is for the amount of dye penetrating into the muscles in connection with their swelling in a solution of phenol red prepared in Ringer's solution without calcium. For more details, see the text.

Second Series: The substantive properties of normal muscles and of muscles in states of increased stimulability were compared in 30 experiments. Experimental muscles were stained with neutral red prepared in Ringer's solution without calcium chloride. For control muscles, the dye was dissolved in normal Ringer's solution. The concentration of the dye in the solutions was 0.025 percent. Staining was continued for 20 minutes. For these experiments, we selected only muscles the stimulability of which to a current of rheobase duration was increased not less than 25 percent as compared with the paired control. The results obrained are shown in Table II (series 2).

A statistically reliable increase in stimulability of muscles caused by the absence of calcium chloride in Ringer's solution is accompanied by a considerable reduction in their ability to bind basic dyes. This reduction is also statistically reliable and its value is 22.8 percent.

Microscopic studies of muscles stained for 20 minutes showed that granules of neutral red were not formed as a rule during this period of time. However, in order to exclude completely the possibility of attributing the reduction of absorption during the phase of heightened stimulability (Troshina showed, in 1956, that a clearly expressed granula formation is seen after staining muscles with neutral red for 30 minutes followed by a period of 30 minutes in Ringer's solution without dye. It is interesting to note that the special experiments of Troshina demonstrated the absence of any differences in the intensity of granule formation in muscles with intensified stimulability (kept in Ringer's solution without calcium) and in muscles with normal stimulability. Intensification of granule formation was noted only during the phase of decreased stimulability.) to suppression of granule formation, we set up a series of experiments in which staining of the muscles was continued only for five minutes. The results of these observations, which are shown in Table 2, No 2, showed that under these conditions, which completely exclude the possibility of granule formation, there was a reliable reduction in absorption which could be attributed only to changes in the absorption properties of the cytoplasm. Hence, it may be considered established that, during the phase of increased stimulability caused by the absence of calcium salts in Ringer's solution, there is a reduction of absorption capacity of muscles with respect to a basic dye -- neutral red.

Third series. It seemed to us important to determine whether there were any changes in the absorption properties of muscles with respect to acid dyes upon increasing their stimulability, since a reduction in the binding powers of

cytoplasm, both for basic and for acid dyes, is sufficient testimony to a reduction in the number of active ionizing acid and for acid dyes, is sufficient testimony to a reduction in the number of active ionizing acid and basic groups on the surface of protein molecules.

As the acid dye we used phenol red (0.03 percent) prepared in normal Ringer's solution. Staining with phenol red of paired sartorius muscles under identical conditions showed that, at one and the same level of stimulability (determined in terms of the constants A and B), no differences in the absorption properties of the two groups of muscles could be detected (Table 2, series 3).

Fourth series. In order to increase the stimulability of muscles we used Ringer's solution without calcium chloride containing 0.03 percent of phenol red. In these experiments we used 37 pairs of muscles. In experimental muscles, the stimulability to a current of rheobase duration increased by an average of 61 percent, and the stimulability to a short stimulus increased very little, but was nonetheless higher than the control. The absorption properties of experimental muscles as compared with the control muscles was reduced by an average of 9.9 percent; however, this figure was not statistically reliable (alpha = 0.815). Nonetheless, on the basis of these experiments, it is not possible to state that the absorption properties of muscles with respect to acid dyes remained unchanged upon increasing the stimulability. A complicating circumstance was the fact that, in the 0.03 percent solution of phenol red prepared in Ringer's solution without calcium chloride, muscles after 20 minutes of staining increased considerably in raw weight (5.5 mg. $\pm$ 0.79 from an average of 10 experiments).

During the same period of time, paired (control) muscles kept in normal Ringer's solution with phenol red of the same concentration did not change in weight (0.01 mg $\pm$ 0.36; average of 10 experiments). The difference in the weight of the control and experimental muscles is completely reliable (alpha equals 0.999). Obviously, due to the swelling of the muscles, greater amounts of dye enter them than enter control muscles. If it be accepted that dye solution of the same concentration as is found in the surrounding medium, enters a muscle in the same way as does water, then tentative calculations show that, with an increase in the weight of the muscle of 5.5 mg due to swelling, the weight of dye is 0.165×10^{-5} grams. Translated into units such as we have been using, this amount corresponds to 0.34 units. In order to establish the magnitude of absorption of dye by the muscle proteins it is necessary to subtract this amount from the total amount obtained experimentally, that is, 0.64 - 0.34 =

2.3 [sic] units. With respect to the magnitude of absorption in controls this comprises 21.5 percent.

Hence, muscles with increased stimulability caused by the action on them of Ringer's solution devoid of calcium are characterized by a considerable reduction of their ability to bind not only basic but also acid dyes.

Fifth series. It would be natural to ask ourselves whether muscle tissue in the presence of increased stimulability possesses in general a lower level of absorption capacity or whether the reduction in absorption which we observed is due to specific changes arising as a result of the absence of calcium chloride in the Ringer's solution. An exhaustive answer to this question may be obtained only from an analysis of the effects on muscles of various agents which increase their stimulability. This will comprise the task of future experiments. In the present work we thought it necessary to determine the presence of tinctorial changes with increases in stimulability of muscles by any method whatsoever. Without wishing to use chemical agents, because of the possibility of their absorptive competition with dyes, we used neutral red in the manner already described, in certain definite concentrations, in order to increase the stimulability of muscles. An increase in stimulability (by 36 percent) occurred with ten-minute staining of sartorius muscles with 0.05 percent solution of neutral red prepared in Ringer's solution without soda. For control muscles, we arranged conditions under which their stimulability did not change despite a ten minute staining with 0.05 percent neutral red. This was achieved by a brief (10-minute) preliminary exposure of the muscles to 4.5 percent ethyl alcohol diluted in Ringer's solution, with subsequent washing in pure Ringer's solution which was changed twice.

Experimental and control muscles were stained simultaneously in one and the same vessel. Stimulability was measured, as in the preceding experiments, directly prior to immersing the muscles in the dye and immediately after staining. For extraction of the dye, we did not use muscles in which stimulability to prolonged stimuli differed from control values by less than 20 percent. In Table II (series 5), the average arithmetical results of changes of the thresholds of stimulability and of absorption properties are presented. The stimulability of control muscles as a result of staining does not change with respect to prolonged stimuli, and decreases only slightly with respect to short stimuli; the stimulability of experimental muscles to a rheobase current, that is, muscles not subjected to preliminary exposure to alcohol, increases by 36 percent over the original level.

Table 3

Changes in the Thresholds of Stimulability and of Absorption Properties of the Sartorius Muscle of Frogs

No II/II	Number of experiments	Conditions of experiments	Time of staining (in min.)	Difference in long threshold of stimulability between experimental and controls (in percentages of the original)	Changes in the absorption of dye in percentage (o/k x 100 - 100%)
1	22	Ringer's solution + neutral red 0,025% (experimental)	20	— 2.4	+ 2.1
	22	Ringer's solution + neutral red 0.025% (control)	20		
2	10	Ringer's solution without calcium chloride + neutral red 0.025% (exp)	5	—32.1	18
	10	Ringer's solution + neutral red 0.025% (control)	5		
3	30	Ringer's solution without calcium chloride + neutral red 0.025% (exp)	20	— 43.1	—22.8
	30	Ringer's solution + neutral red 0.025% (control)	20		
4	20	Ringer's solution + neutral red 0,05% (experimental)	10	—34.8	16.3
	20	Ringer's solution + 4.5% ethyl alcohol for 10 min. Ringer's solution for 10 min; neutral red 0.05% + Ringer's solution (control)	10		
5	10	Ringer's solution + phenol red 0.03% (experimental)	20	1.0	5.0
	10	Ringer's solution + phenol red 0.03% (control)	20		
6	37	Ringer's solution without calcium chloride + phenol red 0.03% (exp)	20	—31.8	0.0 (with a correction factor of 21.5%)*
	37	Ringer's solution + phenol red 0.03% (control)	20		

*Note: Correction for the amount of dye entering the muscles in connection with their swelling in a solution of phenol red prepared in Ringer's solution without calcium. For more details, see text.

The short stimulability of the experimental muscles does not change beyond the limits of statistical reliability, but nonetheless it is higher on the average than in controls. The amount of dye absorbed by muscles with higher stimulability was 16.3 percent less than in controls.

Hence, in this series of experiments, likewise, it was discovered that, given two groups of muscles possessing different stimulabilities to a rheobase current, the group with increased stimulability is characterized by lesser absorption capacities of the substrate with respect to basic dyes than the group possessing lower stimulability.

Summing up the results of the present work, we set up Table 3, from which the relations of the threshold of rheobase stimulability and of absorption properties of sartorius muscles are apparent. From the table it follows that, with an original rheobase stimulability of equal magnitude for both paired muscles, there were no essential differences with respect to absorption properties, either (Table 3, No 1 and 5). In cases in which there was an increase in stimulability, the absorption capacities of muscles were reduced by 16 to 20 percent as compared withthe normal, following staining either with basic or with acid dyes.

Conclusions

The interrelationship between stimulability and tinctorial properties established in our experiments was also observed by V. P. Trochina (1957) in studying the survival of satorius muscles of the frog in cold. It may be suggested that, under these conditions, a weakening of absorption is the result of a decrease in the number of acid and basic ionizing groups on the surface of protein molecules of the cytoplasm, or, in other words, the phase of increased stimulability corresponds to an intensification of the processes of renativation of ctyoplasmic proteins. In support of this also is the work of Kondrashova (1954), who observed during the phase of increased stimulability of muscles an intensification of the synthesis of high energy phosphate compounds (an increase in the content of phosphocreatine and adenosine triphosphoric acid). On the contrary, with a reduction of stimulability, she was able to detect a reduction in the synthesis of high energy phosphate compounds. However, for final conclusions on the nature of the changes in the substrate with increases in stimulability, we have at our disposal very little factual material. Further studies will enable us to establish the reliability of the hypothesis that, during the phase of increased stimulability, there are changes in the protein substrate in the direction of renativation, and that there are interrelationships between the substantive

and functional characteristics of tissues in the early phases of parabiotic processes.

Conclusions

1. With an increase of 30 to 80 percent in the stimulability of muscles to a current of rheobase duration, the absorption by such muscles of basic (neutral red) and acid (phenol red) dyes decreases by 16 to 22 percent as compared with control muscles.

2. For an explanation of these facts, we suggest that, during the phase of increased stimulability, there is an intensification of the processes of renativation of cytoplasmic proteins.

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Changes in the Functional and Substantive Properties of Muscles of Frogs, Depending on the Length of Time They are Kept in Ringer's Solution

N. A. Vinogradova

The Laboratory of Cellular Physiology of the Institute of Cytology of the Academy of Sciences USSR, Leningrad

The task of the present work was to investigate the functional and substantive properties of isolated muscles kept in Ringer's Solution at room temperature. This work was of importance since isolated muscles kept in Ringer's solution ordinarily serve as "controls" in experiments on the effects of different agents on muscle tissue.

Methods

The experiments were carried out using the sartorius muscles of grass frogs during the autumn and winter period. Studies were made of contractions, resting currents, stimulability, resistance, and the ability of muscles to bind vital dyes after being kept for long periods of time in Ringer's solution. Isolated muscles were weighed and suspended by their tendons in vessels of identical size containing Ringer's solution. They were kept in this position for definite periods of time at temperatures from 16 to 22°C. Determination of various indices of the state of the muscles were carried out at 1, 3, 4, 7, 13, 25, 37, 49 and 73 hours after isolation of the muscles. As controls, we used the results of measurements carried out one hour after the isolation of the muscles.

Recording of contractions was made on a slowly rotating drum (rate of rotating was one time per twenty-four hours). A Julien lever was used. Its stress on the muscle was equal to 100 mgm. In all series of experiments in which the Julien lever was not used, a 100 mgm. weight was suspended to the end of the muscle.

For the recording of resting currents, use was made of a mercury-calomel electrode with agar switches [?] joined with a mirror galvanometer. At definite intervals of time, the muscles were taken from the Ringer's solution and placed in a moisture chamber for determination of differences of potential. Measurements were continued for 48 to 60 minutes at intervals of five minutes. Since the difference in potentials of sartorius muscles, according to the findings of D. N. Nasonov and V. Ya. Aleksandrov (1944) and according to our preliminary observations, first increases and then declines,

we used the maximum values for comparison of the magnitudes of resting current from experimental and control muscles.

For a characterization of the stimulability of muscles we determined the values of the constant "a" (short threshold) and "b" (long threshold) of the Gorveg-Veys formula, as suggested by D. N. Nasonov and D. L. Rozental' (1953). Determinations were carried out with the aid of a condensor chronaximeter, with a capacity of 0.01 to 600 microfarads and a shunt of 100 ohms, at voltage intervals from 1.5 to 150 volts. The values of the constants (a) and (b) were expressed as percentages of the control values taken as 100 percent.

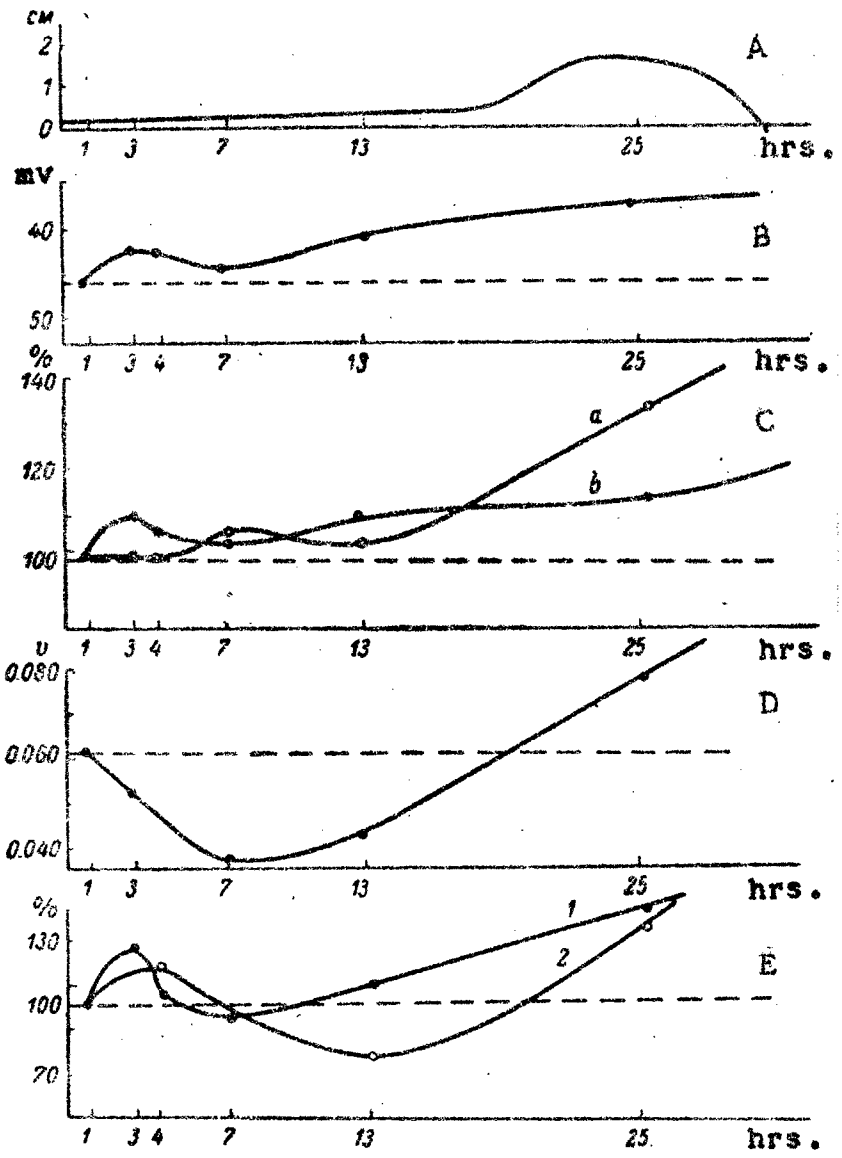
For determination of the resistance of the muscles we used a thermal stimulus. The time of survival of muscles in Ringer's solution heated to 38°, or the rate of death of muscles under these conditions (v = 1°time of survival), were used for comparison of the resistance of muscles kept in Ringer's solution for different periods of time. The criterion of death of the muscles was the absence of contractions in response to stimulation with an electric current from a Dubois-Raymond inductor.

The absorption capacities of muscles were studied with respect to basic and acid dyes. As the basic dye, we used 0.01 percent neutral red prepared in Ringer's solution without soda; as the acid dye, we used 0.05 percent phenol red prepared in Ringer's solution. The muscles were stained for an hour at 17°C. The amount of dye absorbed was expressed as a percentage of the control values.

The material which we obtained was processed statistically. The results of the experimental finds are shown in the table and in the figure.

Results of Experiments

In all experiments, death of muscles in Ringer's solution was always accompanied by contraction. In the figure (A) are shown the myograms of muscular contractions (separate experiment). In the majority of cases, contractions appeared 12 to 18 hours after isolation of the muscles. By 18 to 24 hours it had reached a maximum, and by 37 hours the muscles began to become flaccid. Sometimes contraction appeared by the end of the second day, and flaccidity ensued only on the third day.



Changes in the Functional and Substantive Properties of Muscles Depending on the Period of Time for Which They are Kept in Ringer's Solution.

Along the abscissa -- time of keeping muscles in Ringer's solution (in hours); along the ordinate; A -- height of muscular contraction (in centimeters); B -- magnitude of resting current (in millivolts); C -- magnitude of thresholds of stimulability (a -- short threshold, b -- long threshold), expressed in percentages of control values; D -- rate of onset of non-stimulability of muscles (V) -- upon exposure to temperature of 38°C (v = 1°time of survival); E -- amount of dye absorbed, in percentages of control (1 -- neutral red,

Changes in Functional and Substantative Properties of Muscles Depending on the Time for Which They are Kept in Ringer's Solution

Time for which the muscles are kept in Ringer's solution (in hours)	Value of resting current (in millivolts)		Magnitude of thresholds of stimulability (in percentage of control)		
	No of exper.	M ± m	No of exper.	b M ± m	a M ± m
1 (control)	15	45.4 ± 0.7	150	100	100
3	14	42.9 ± 0.8	24	110.7 ± 2.7	100.8 ± 4.3
4	15	43.0 ± 0.6	29	106.2 ± 2.2	100.2 ± 2.2
7	16	44.2 ± 0.6	30	103.9 ± 1.6	106.0 ± 3.0
13	15	41.8 ± 0.6	17	109.8 ± 4.8	103.0 ± 3.0
25	16	38.9 ± 0.9	30	113.5 ± 3.4	134.0 ± 6.0
37	8	9.3 ± 0.7	12	134.0 ± 4.5	208.0 ± 10.4
49	—	—	8	199.0 ± 21.0	433.0 ± 50.0
72	—	—	—	—	—

Resistance of muscles upon exposure to a temperature of 38°C			The absorption of neutral red (% of control)		The absorption of phenol red (in % of control)	
No of exper.	Time of survival of muscles (in min.) M ± m	Rapidity of onset of non-stimulability (1/time)	No of exper.	M ± m	No of exper.	M ± m
16	16.4 ± 0.9	0.061	59	100	58	100
16	19.7 ± 0.7	0.051	8	126.0 ± 7.7	—	—
—	—	—	7	103.7 ± 3.7	8	117.0 ± 5.5
20	23.4 ± 1.0	0.038	12	92.1 ± 5.7	6	98.0 ± 6.6
12	22.3 ± 0.6	0.044	8	106.0 ± 3.5	12	76.0 ± 4.9
12	17.7 ± 1.0	0.057	8	142.1 ± 11.1	12	137.0 ± 7.5
12	12.7 ± 1.0	0.079	—	—	—	—
8	6.3 ± 1.1	0.159	8	275.4 ± 15.9	11	470.0 ± 46.0
—	—	—	8	629.1 ± 75.4	—	—

The value of the resting current, as can be seen from the table and the figure (B), begins to change in the first hours after the isolation of the muscles. By the end of the third hour in Ringer's solution, the difference in potential is reduced by 2.5 millivolts (reliability:

alpha = 0.993). When kept for longer periods of time in Ringer's solution, the decline in the resting current is retarded and there is even a slight increase which, however, is not statistically reliable. In order to test the reliability of the increase in the difference of potentials, we set up a special series of experiments in which paired muscles were compared, one of which was kept in Ringer's solution for three hours and another for seven hours. In five cases out of six, the resting current of muscles kept in Ringer's solution for seven hours was higher than the currents of muscles kept in Ringer's solution for three hours. The difference, on the average, was 1.8 millivolts (alpha = = 0.977), that is, it was statistically reliable. With longer periods of time in Ringer's solution, the difference in potentials was reduced, and by the end of the second day it was no longer possible to detect any difference. These findings permit us to conclude that the magnitude of the resting current of muscles is tri-phasic.

A triphasic change could be seen also in constant (b) (Table and Figure, (B)). During the first three hours in Ringer's solution the thresholds to long stimuli increased to 110.7 percent as compared with controls. By seven hours of incubation the magnitude of threshold had reverted to the original level (alpha = 0.986). By 25 hours, (b) increased to 113 percent (alpha _ 0.996), and from then on the threshold progressively increased.

For short stimuli, thresholds did not change during the first three hours (Figure, (V)). A certain tendency to increase was seen after seven hours in Ringer's solution (an increase to 106 percent, alpha = 0.955). By 13 hours the magnitude of the threshold was 103 percent (alpha = 0.788), and by 25 hours the threshold had increased to 134 percent (alpha = 0.999).

The resistance of the muscles, as seen from the Figure (D), increased during the first seven hours. The time of survival of muscles at a temperature of 38° by this time was ten minutes longer than that of the controls (alpha = 0.999). With longer times in Ringer's solution, the resistance of the muscles rapidly decreased.

The absorption capacities of muscles with respect to basic and acid dyes showed a triphasic change, likewise. As can be seen from the Figure (E), during the first hours the level of absorption increased: for neutral red, by three hours, the magnitude of absorption was 126 percent; for phenol red, by four hours, it was 117 percent. After seven hours in Ringer's solution, the absorption capacity of muscles reverted to the original level. By 13 hours of incubation, the amount of neutral red absorbed began to increase, whereas for

phenol red the absorption capacity was reduced to 76 percent (alpha = 0.999). By 25 hours, the amount of dye absorbed had increased considerably: for neutral red it was 142.1 per cent (alpha = 0.999), and for phenol red it was 135 percent (alpha = 0.999). With longer periods of incubation, the level of absorption increased.

The fact that similar changes were seen in absorption both of basic and of acid dyes leads us to believe that changes in absorption are not connected with changes in pH, but are the result of alteration in the muscle proteins.

Discussion

These data testify to the fact that, immediately after isolation of muscles from the organism, their functional and substantative properties begin to change. This change is of a phasic character: during the first three hours the resting current and the stimulability decrease, and the absorption capacities and resistance increase, whereas by seven hours of incubation, the resting current increases slightly, the stimulability and absorption properties return to the original level; the resistance, however, remains elevated. By 25 hours the resting current, stimulability, and resistance are markedly reduced, while the absorption properties, on the other hand, are increased. At this same time, the majority of muscles are in a state of maximum contractions.

Phasic changes in stimulability and absorption properties were also observed by V. P. Troshina (1957) in isolated frog muscles kept in Ringer's solution at temperatures approaching 0°C.

Upon comparing the experimental material which we obtained with data taken from the literature (Nasonov, 1948; Nasonov and Suzdal'skaya, 1948; Rosental' 1948; Ushakov, 1954; Dzhamusova, 1958), it is possible to conclude that during the process of being kept in Ringer's solution, muscles enter a state of stable local stimulation, which subsequently leads to death.

A number of works have been devoted to an explanation of the causes of death of isolated muscles, in which it has been shown that there is a gradual loss of glycogen reserves in isolated muscles (Hoet and Marks, 1926; Bate-Smith and Bendall, 1949; Moreva, 1954; and Tank, 1954) and the content of glycogen becomes so negligible that it is impossible to sustain the amount of ATP at a normal level (Bate-Smith and Bendall, 1949; Bendall, 1951). The reduction in the concentration of ATP may be the direct cause of denaturative changes in the muscle proteins (Szent-Gyorgy, 1947).

Starting with the protein theory of injury and stimu-

lation as expounded by D. N. Nasonov and D. Ya. Aleksandrov (1940), it may be assumed that the phasic changes in the functional and substantive indices of the state of isolated muscles kept in Ringer's solution observed in our experiments are caused by denaturative changes is muscle proteins.

I wish to express my gratitude to B. P. Ushakov for his supervision.

Conclusions

1. Studies were made of functional and substantive changes in isolated sartorius muscles of frogs kept for long periods of time in Ringer's solution at room temperature.

2. Functional and substantive properties begin to change immediately after isolation of the muscles from the organism, this change being of a phasic character. The magnitude of the resting current, the absorption capacities, and the long and short stimulability of muscles change in a triphasic fashion, whereas changes in resistance are biphasic.

3. Death of muscles in a Ringer's solution is always accompanied by contraction, following the development of which the stimulability of the muscle is markedly reduced, the magnitude of the resting current decreases, the resistance declines, and the absorption capacity increases.

4. These results together with the findings from the literature permit us to conclude that, in isolated muscles, during the process of dying in Ringer's solution, a state of stable local stimulation develops.

5. Strating with the protein theory of stimulation and injury of D. N. Nasonov and V. Ya. Aleksandrov, it may be assumed that the phasic changes in functional and substantive indices which we observed have their explanation in the denaturative changes of muscle proteins.

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Changes in the Transparency of the Dermosternal Muscle of Frogs Upon Exposure to Different Modifying Influences

A. A. Kuskaina

Department of Human and Animal Physiology of Leningrad University

The concept that parabiosis is a phasic process, in the development of which an important role is played by stimulation, has been reinforced by findings concerning biphasic substantive changes of tissues in the course of alteration. The demonstration of phasicity in these substantative changes has been facilitated by the use of quantitative methods of investigation. Therefore, primary emphasis has been given to descriptions of biphasic changes in viscosity of cytoplasm in the presence of various influences, as reported in the monograph of Heilbrunn (1958). Biphasic changes in the absorption of vital dyes (reduction, with subsequent increase) have been noted by Z. F. Zarakoskiy and S. V. Levin (1950, 1952, 1953), A. V. Zhirmunskiy (1954, 1958), B. P. Ushakov (1954), N. V. Golovina (1955) and others. Substantive changes may be detected in the development of turbidity of tissues, which testifies to a reduction in the degree of dispersion of colloids in the cytoplasm, which is one of the basic signs of paranecrosis (Nasonov and Aleksandrov, 1940). Therefore, quantitative studies of changes of translucency in the course of alteration of tissues have become of central interest. Preliminary findings in this direction were obtained in the laboratory of L. L. Vasil'yev and A. A. Dobashina (1947). At the suggestion of L. L. Vasil'yev, we undertook studies of the changes in translucency of muscles upon the development of a parabiotic process.

Methods

Studies of the optical properties, in particular measurements of the transparency of tissues, are expedient by virtue of the fact that a light beam of small intensity, acting on the tissues for the short period of time necessary for measurement, does not influence the functional state of the object and therefore does not introduce any significant distortions in the results of the experiment. Optical methods are quite sensitive and, moreover, have permitted us to make measurements on one and the same preparation from the very onset of alteration.

Experiments were set up on the dermo-sternal muscle of the frog, which consists of one to two layers of muscle

fibers which are uniform in thickness and almost colorless. The very slight thickness of the muscle permits quick penetration of chemical agents into it and virtually simultaneous alteration of all the component fibers. Parabiosis of the muscle was caused by pressure, by the application of a direct current, and by solutions of caffeine, chloral hydrate, ethyl alcohol, potassium chloride, and calcium chloride. All these solutions were prepared in Ringer's solution. The choice of altering agents was determined by the fact that the parabiotic process which they elicit has been well studied in the laboratory of N. Ye. Vvedenskiy.

The muscles were placed in a special glass container fitted with a profusion apparatus which permitted quick replacement of the Ringer's solution in the container with a solution of modifying agent. A beam of light of constant intensity was passed through the container with the muscles. Measurements were made of the original strength of the light passing through the container holding the muscles, as well as of its changes in the process of alteration. For measuring the strength of the light we used the ionic contrast apparatus IK-1, one division of the scale of which corresponded to 1×10^{-5} international candles. The apparatus consisted of the photomultiplier FEU-1, a rectifier, and an amplifier in bridge assemblage. In the course of the experiment we measured turbidity in percentage of the original level. Control experiments showed that within six to seven hours muscles kept in isometric conditions become turbid without any outside influence. Within the first hour after preparation, there was a negligible variation in the translucency of the muscles. Therefore, the experiments were begun within 60 to 90 minutes after preparation and were continued not longer than five to five and a half hours. With this, the variations in translucency in control muscles did not exceed two to four percent of the original level. In all, we ran about 800 experiments.

Changes in the Translucency of Muscles Following Compression and Following Exposure to Chemical Agents

The most clear-cut results were obtained with compression and with the use of non-electrolytes. A 30-second compression with the aid of a special compressor, described in the work of M. A. Rayevskaya (1948) was applied to a thin ribbon of tissue measuring an average of 10 square millimeters in area. In distinction from the experiments with chemical modifying agents, measurements of translucency were carried out after cessation of the action of the modifying agents. This set-up of the experiments was justified by the fact that

substantive changes in muscle tissue following mechanical action continue to develop during the course of many hours after discontinuation of compression. (Rayevskaya, 1948). The curves showing the changes in translucency with the passage of time as the result of compression of different strengths (Fig. 1A) testify to the fact that translucency of muscles undergoes a biphasic change. The average values for the changes in translucency, on the basis of which the graphs were set up, both here and in a subsequent series of experiments, are statistically reliable. Weak compression (200 gm/sq. cm.) caused an increase in translucency with subsequent restoration of the original level, whereas strong compression (2000 gm/sq. cm.) caused only a turbidity of the muscles. Following compressions of 500 and 1000 gm/sq. cm., an original increase in transparency was superseded by an irreversible diminution in transparency. As shown by special experiments for measurements of the thickness of muscles in the course of alteration, the original increase in transparency cannot be attributed to loosening of the tissues, since the original thickness of the muscles was re-established within three to seven minutes after application of compression.

Changes in translucency of muscles under the influence of caffeine are also distinctly biphasic. In Fig. 1B, curves are shown which illustrate the changes in the transparency of muscles with the passage of time following exposure to solutions of caffeine of differing concentrations. An 0.025 percent solution of caffeine causes an increase in transparency, and delays for three to five hours the onset of spontaneous turbidity of the muscles. An increase in concentration leads to a biphasic change in translucency. Caffeine in concentrations of 0.2 and 0.4 percent causes turbidity of muscles without preliminary increase in translucency. With this, the rate of increase in turbidity is greater with the more concentrated solutions. Replacement of the caffeine solution with pure Ringer's solution leads to quick restoration of the original level of transparency in cases in which the transparency of the altered muscle was greater than the original. Turbidity of muscles caused by exposure to 0.05, 0.1, and 0.2 percent solutions of caffeine is also reversible if they are washed within five to 15 minutes after the onset of turbidity. In this, a phase of increased translucency precedes the restoration of the original level of translucency.

We were able to find the biphasic character of changes in the degree of dispersion of colloids of muscles upon alteration by caffeine reported only in one study (Baeyer and Muralt, 1934). These authors believe that the 0.09 percent solution of caffeine which they used caused immediate turbidity of

muscles; however, from the protocol of their experiments, it would seem that the turbidity was preceded by an increase in translucency during the first 15 to 20 minutes of exposure to the altering agent.

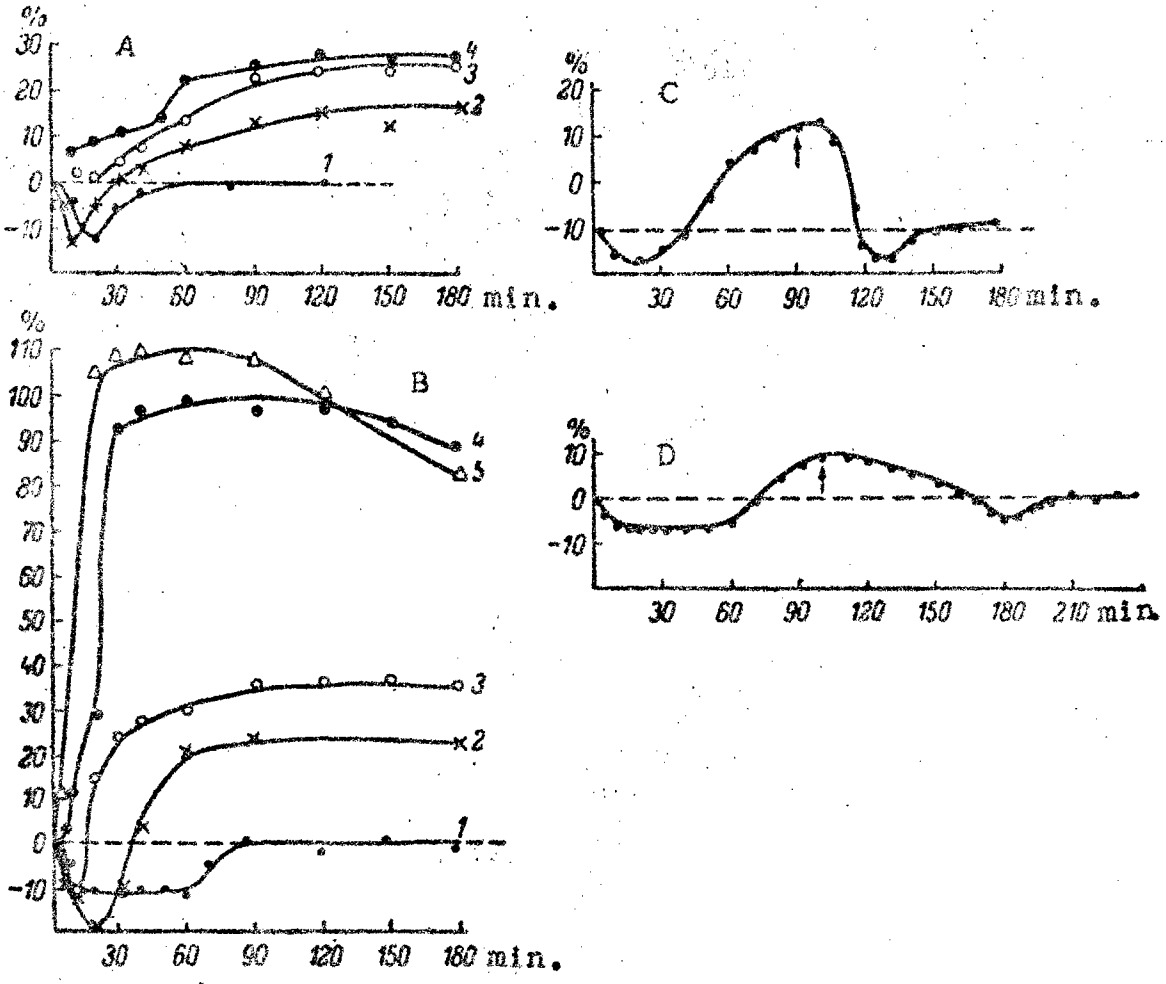

Fig. 1. Changes in the Turbidity of Muscles Upon Alteration by Compression and by Solutions of Non-electrolytes.

A -- After a 30-second compression with strengths of: 1-200 gm/sq. cm, 3-1000 gm/sq. cm. and 4-2000 gm/sq. cm.; B -- upon alteration by solutions of caffeine: 1-0.025%, 2-0.05%, 3-0.1%, 4-0.2%, and 5-0.4%; C -- Upon Chloral hydrate. Along the abscissa -- time from the beginning of alteration, along the ordinate -- changes in turbidity in percentage of the original level. Arrows indicate washing; dotted lines indicate the original level of transparency. A and B -- the families of curves for different doses of a single agent,

in which each point represents the arithmetic average of 5 to 12 measurements.

We also used ethyl alcohol and chloral hydrate as modifying agents. Among the numerous works devoted to the effects of narcotics on the physical-chemical properties of cytoplasm, there were also studies in which description was given of a biphasic character of the paranecrotic reaction to modification by alcohol and chloral hydrate. Romanov (1949) described a phase of reduction in the absorption properties of nerve cells in the course of restoration of normal absorption following exposure to alcohol. A phase of reduction in the staining properties of sartorius muscle during the first 30 minutes of exposure to 4.5 percent alcohol, and during the first 60 minutes of exposure to 0.25 percent chloral hydrate, has been described by B. P. Ushakov (1954). Since the reduction in absorption properties coincided in time with an increase in the rheobase and with a shortening of the chronaxie, then this, in the opinion of the author, is a substantive expression of the first phase of the parabiotic process.

In 19 of the 22 experiments which we ran using 4.5 percent ethyl alcohol, and in all 20 experiments with 0.25 percent chloral hydrate, biphasic changes developed in the translucency of muscles (Fig. 1, C and Fig. 1, D). The initial increase in translucency lasted for 30 to 40 minutes in the case of alcohol and for 50 to 70 minutes following exposure to chloral hydrate, after which turbidity of the muscles developed. A phase of increased turbidity may also be seen in the course of restoration of the original translucency when the altering agent is washed away.

It was of particular interest to us to study transparency upon exposing muscles to potassium chloride and calcium chloride, since the parabiotic process caused by ions of calcium is characterized by a considerable predominence of the first phase, and that caused by ions of potassium by a predominence of the second phase. In dark field studies of isolated nerve cells exposed to the chloride salts of potassium and calcium (Ettisch and Jockims, 1927; Goldenberg and Vasil'yev, 1929), it was shown that potassium reduces and calcium increases transparency. However, in both works, only isotonic solutions of salts prepared in distilled water were used, and the photographs shown in the articles were made not earlier than an hour after the onset of exposure of the muscles to the altering agents. Under these conditions, it is difficult to detect phasicity in the changes of dispersion. I. Ye. Kamnev (1939, 1948) showed that the complex of paranecrotic changes in muscle tissue, including luminescence

in a dark field, may be caused by the action of potassium and calcium, that is, an increase in light scattering is characteristic of the action of both of these agents.

The results of our experiments on the translucency of muscles following exposure to the chloride salts of potassium and calcium are shown in Figure 2, A and B. Upon exposure to weak and moderate concentrations of potassium chloride, the translucency of muscles changes in the same way as in solutions of the non-electrolytes which we studied. A slight increase in transparency is seen upon exposure to 0.05 and 0.1 percent solutions, whereas biphasic changes in translucency are elicited by 0.2 percent solutions of potassium chloride. Beginning with the 0.5 percent solution, there is a secondary translucency of muscles after a 1.5-hour alteration. A similar secondary translucency is seen upon exposure of muscles to 0.8 and to 1 percent solutions. In cases in which there is a secondary translucency after turbidity, it is not possible to detect the phase of increased translucency at the beginning of alteration. Some evidence concerning the nature of the secondary translucency is given by measurements of translucency upon washing the muscles with Ringer's solution. If this washing is begun during the phase of primary translucency, the original level of translucency is re-established very quickly (Fig. 2, C). The washing begun early in the phase of turbidity also almost always leads to restoration of the original level of transparency, the establishment of which is preceded by a phase of increased transparency (Fig. 2, D). Washing begun during the time of secondary translucency leads to an abrupt and marked turbidity of the muscles (Fig. 2, E) Obviously, the dispersing influence of potassium chloride on colloid particles masks the turbidity associated with the denaturation of the muscle proteins. This turbidity appears upon washing. The development of turbidity after removal of the dispersing agent has been described in a number of works (Lewis, 1927; Rumyantsev, 1932; Kamnev, 1939).

Similar changes in translucency are caused by exposure to calcium chloride, which we used in a range of concentrations from 0.25 to 8 percent (Fig. 2, B). In this case, weak solutions (0.5 percent) also cause an increase in translucency; however, this is distinguished by considerably greater duration (six to seven hours). Beginning with one percent concentration, calcium chloride causes turbidity of the muscles, which is followed by a secondary translucency in the case of four and eight percent solutions. Just as in the case of modification by potassium chloride, washing the muscles in pure Ringer's solution during the phase of secondary translucency leads to quick and irreversible turbidity (Fig. 2, F).

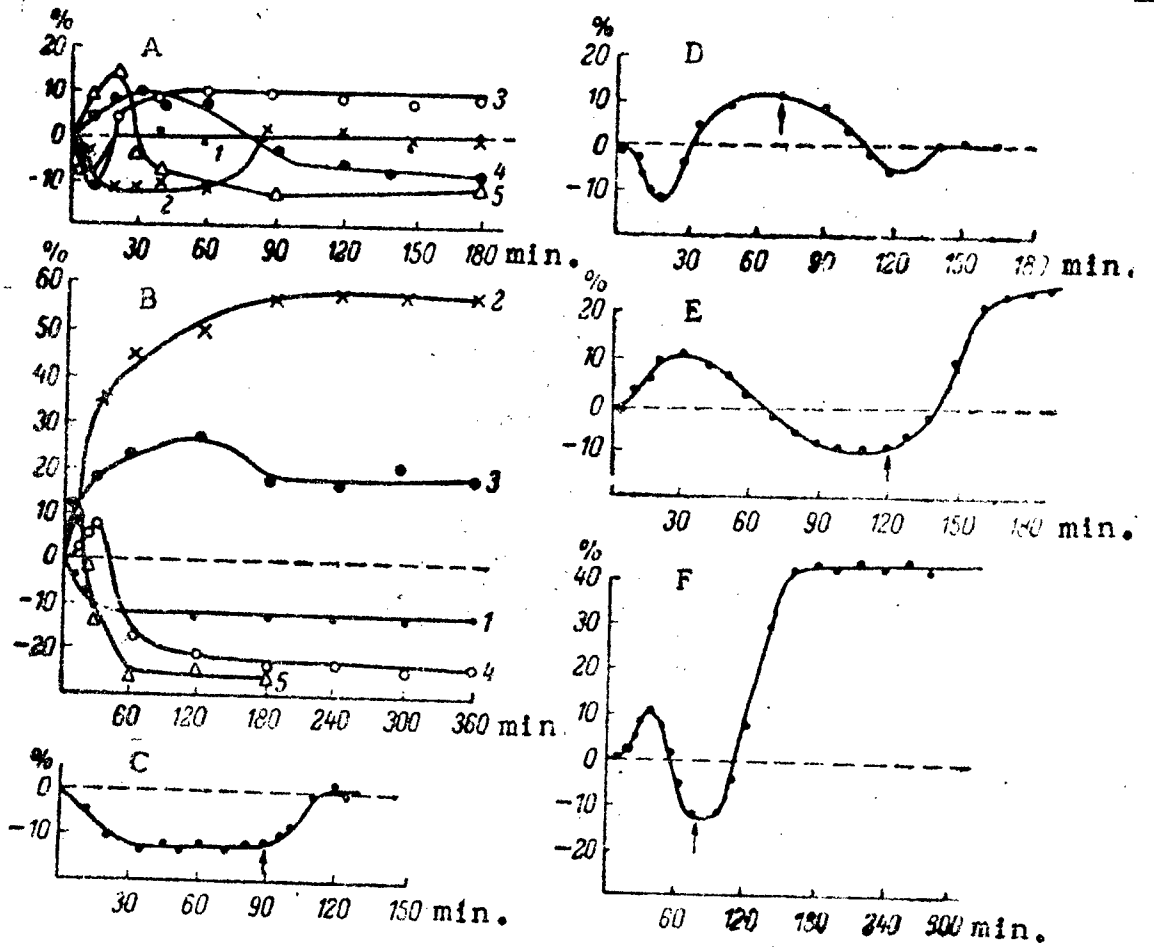

Fig. 2. Changes in the Turbidity of Muscles Upon Exposure to Potassium Chloride and Calcium Chloride.

A -- Upon alteration by solutions of potassium chloride: 1 - 0.05%, 2 - 0.1%, 3 - 0.2%, 4 - 0.5%, and 5 - 1%; B -- Upon alteration by solutions of calcium chloride: 1 - 0.5%, 2 - 1%, 3 - 2%, 4 - 4%, and 5 - 8%; C, D, E and F -- Reestablishment of the original transparency of altered muscles: C -- After alteration by 0.1% potassium chloride, D -- 0.2% potassium chloride, E -- 0.5% potassium chloride, F -- 4% calcium chloride. The remaining designations are the same as in Fig. 1.

An analysis of the changes in the transparency of muscles under the influence of electrolytes is complicated by the fact that the action of electrolytes on tissues is very complex. One is dealing not only with changes associated with denaturation of tissue proteins, but also by the effects of the salts on the charge and on the degree of

hydration of colloid particles, as well as with the complicating influence of hypertonicity of salt solutions. In various experiments, one or another of these factors may be of preponderant influence.

Exposure to weak solutions of chemical substances in concentrations causing an increase in translucency of muscles considerably postpones the onset of their spontaneous turbidity. It is interesting to compare this fact with the findings of M. B. Kiro (1954), who showed that weak solutions of modifying agents caused a prolongation of the survival time of muscles as compared with their survival in pure Ringer's solution. Concentrations of ethyl alcohol, chloral hydrate, and calcium chloride causing an increase in the survival time of muscles as compared with survival in Ringer's solution, proved to be the same as the concentrations causing an initial increase in translucency and postponing the onset of spontaneous turbidity of muscles. In our experiments, these concentrations were as follows: for alcohol - 4.5 percent, for chloral hydrate - 0.25 percent, for calcium chloride - 0.5 percent; in the experiments of M. B. Kiro on the sartorius muscle, the concentrations were as follows: 4-5 percent for alcohol, 0.05-0.2 percent for chloral hydrate, and 0.3-0.4 percent for calcium chloride.

Changes in Translucency Upon Exposure to a Direct Current

The effects of direct current as a modifying agent on living substance have been the object of study of an extensive and, to a considerable extent, contradictory literature. There are conflicting data concerning the problem of changes in translucency of tissues in the region of application of the anode and cathode. Kamnev (1939, 1941, 1949) showed that the passage of a direct current through muscle and cornea leads to the development of a paranecrotic reaction in the area of application of both poles. In the area of the anode, changes in the staining properties and in luminescence in the dark field (which testifies to a reduction of the dispersion of colloids) developed with currents of great strength and long application to a greater extent than in the area of application of the cathode. Conflicting results (an increase in the degree of dispersion of colloids at the cathode and a reduction at the anode) have been obtained by Tobias and his associates in non-vertebrate nerves (Tobias, 1950, 1952; Tobias andSolomon 1950, and others). Despite this fact, both Kamnev and Tobias consider the chief causes of changes in translucency to be hypotonia in the cathode area and hypertonia in the anode area. Tobias believes that entry of water into the cell leads to an increase in the degree of dispersion

of ctyoplasmic colloids, while Komnev believes that there is a reduction in dispersion under the influence of hypotonicity. In the opinion of Kamnev, an excess of water in the cell reduces the concentration of the salts which maintain the necessary level of dispersion of protein colloids under normal conditions.

Our findings confirm Kamnev's data; the use of the quantitative method of study has, moreover, enabled us to discover phasic changes in translucency upon exposure of the tissues to a direct current. Current of a strength of 0.017 to 2.0 milliamps was applied to a muscle with the aid of nonpolarizing electrodes (Zn - ZnO_4). Two woolen threads moistened with Ringer's solution and measuring 0.2 mm. in diameter were applied to a muscle at a distance of 7 mm. from each other, perpendicular to the long axis of the muscle. In distinction from experiments using chemical modifying agents, we were unable to conduct measurements during the time of action of the current. Therefore, polarization was effectuated for periods of 1, 5, 10, 20, 40 and 60 minutes. Immediately after discontinuing the current, the electrodes were removed and the translucency under the anode and under the cathode was measured. Each experiment yielded, for the anode and the cathode, only one figure characterizing the action of a current of given strength over a given interval of time. The curves showing the dependency of translucency on time of action of the current are constructed on the basis of 552 measurements.

In the area of application of the anode, biphasic changes were observed in translucency which were similar to those obtained following exposure to chemical altering agents (Fig. 3, A). A slight increase in translucency appeared after 40 minutes of application of a current of 0.067 milliamps or after ten minutes of administration of a current of 0.125 milliamps. A stronger current (0.25 milliamps) caused biphasic changes in translucency. Further intensification of the current led to turbidity of muscles without an initial increase in translucency.

In the area of application of the cathode (Fig. 3, B) turbidity appeared even with a current of 0.034 milliamps. There was no increase in translucency at the beginning of the action, which, perhaps, is due to the imperfection of our methods. The probability of this explanation is confirmed by the fact that, upon the action of the cathode, the primary phase of the parabiotic process also is very mild and brief (Golikov and Merkulov, 1935). The absence of any signigicant differences in the effects of the two poles on the stimulability of the nerve can be demonstrated only under special conditions: at low room temperatures and with brief

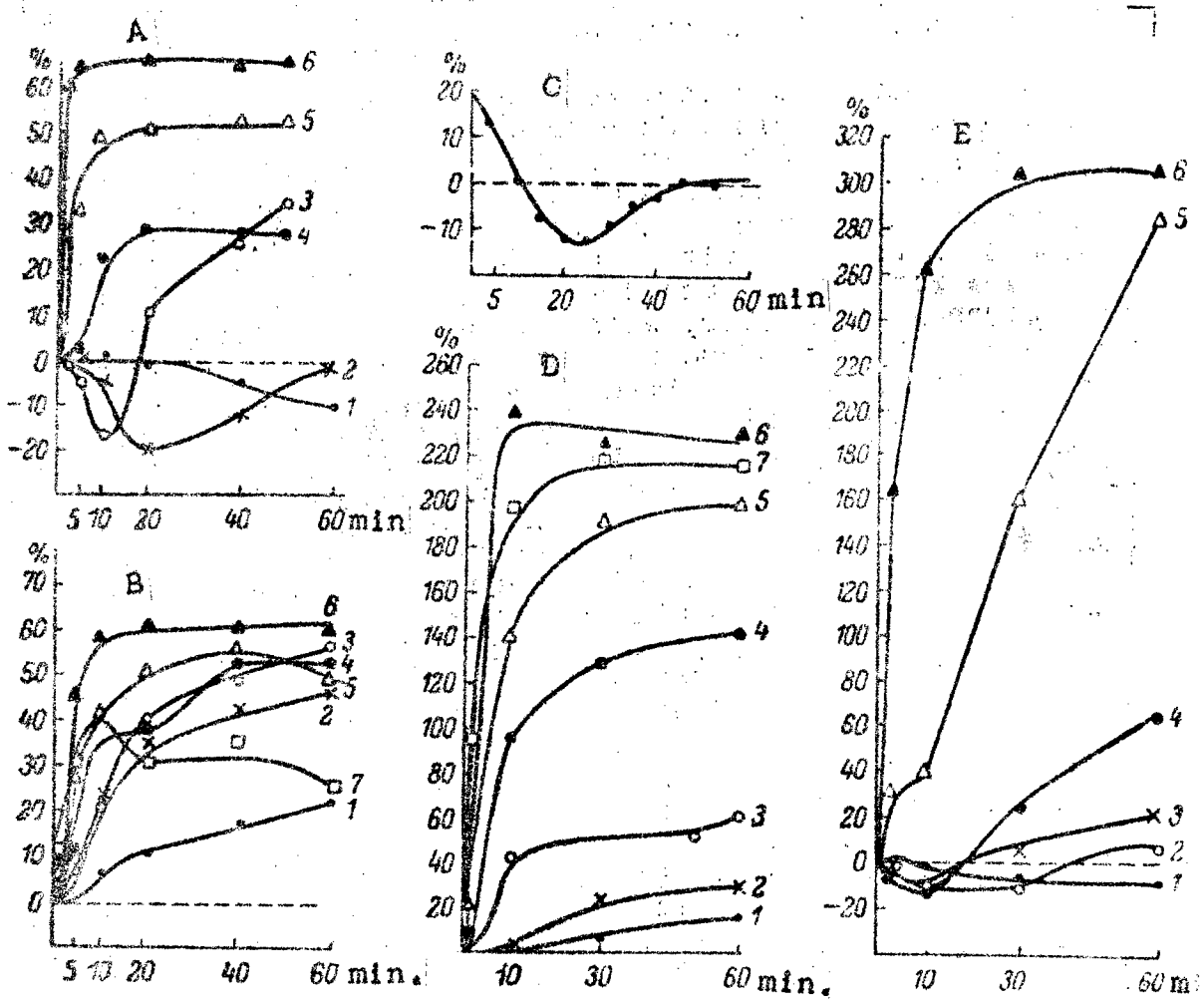

Fig. 3. Changes in the Turbidity of Muscles Upon Administration of a Direct Current.

A -- in the area of application of the anode: 1 -- strength of current 0.067 ma., 2 -- 0.125 ma., 3 -- 0.25 ma., 4 -- 0.5 ma., 5 -- 1.0 ma., 6 -- 2.0 ma.; B -- in the area of application of the cathode: 1 -- strength of current 0.034 ma., 2 -- 0.067 ma., 3 -- 0.125 ma., 4 -- 0.25 ma., 5 -- 0.5 ma., 6 -- 1.0 ma., 7 -- 2.0 ma.; C -- restoration of the original translucency of muscles after discontinuation of polarization of it with the cathode: strength of current 0.5 ma., time of application five minutes; B -- changes in translucency of cornea in the area of application of the cathode: 1 -- strength of current 0.015 ma., 2 -- 0.06 ma., 3 -- 0.12 ma., 4 -- 0.25 ma., 5 -- 0.5 ma., 6 -- 1.0 ma., 7 -- 2.0 ma.; E -- changes in translucency of cornea in the area of application of the anode: 1 -- strength of current 0.12

ma., 2 -- 0.25 ma., 3 -- 0.5 ma., 4 -- 1.0 ma., 6 -- 4.0 ma. Remaining designations same as in Fig. 1.

exposures to a very weak current (Ushakov, Averbakh, Suzdal'skaya, Troshina, Cherepanova, 1953). An increase in translucency as the result of the action of the cathode is seen after discontinuation of the current and prior to the development of primary translucency (Fig. 3, C). It is interesting to note that, with a brief exposure to a weak current, there are rather pronounced individual differences in the transparency of muscles, whereas with a stronger and more prolonged current, these differences become equalized and the results of the experiments become more constant.

The reversibility of changes caused by polarization depends on the strength of the current and on the duration of exposure, and reversal of the direction of the current accelerates reversion of translucency to its original level (Kamnev 1941). Our experiments completely confirmed this fact. The restoration of the original translucency of the muscles, the light transmission of which was increased by the action of the anode, may be considerably accelerated by the application of a cathode. Upon polarization by a cathode, which causes turbidity which is spontaneously reversible, a change in the direction of the current accelerates the process of restoration by a factor of three to six times. Prolonged (one to one and a half hours) application of the anode to the muscle in a state of cathode turbidity which is not spontaneously reversible (strength of current 1.0 ma. for more than 40 minutes), also leads to restoration of the translucency. The action of the cathode for more than ten minutes under conditions in which the current exceeds 2.0 ma. causes turbidity which does not disappear either spontaneously or upon anodization. The intensity and duration of action of the current necessary for restoration of the original translucency depend on the depth of the turbidity. The greater the changes in translucency, the stronger and more prolonged must be the action of the anodes. However, the strength of the restorative current must be less than that of the altering current. Turbidity caused by the action of the anode does not disappear upon the action of the cathode.

Paranecrosis is a non-specific reaction of any cell to external influences (Nasonov and Aleksandrov, 1940). From this point of view, we were interested in determining whether non-conducting tissues would react to the administration of an electric current with the same phasic changes in translucency as muscles.

As our experimental object we selected the cat cornea; the turbidity of corneas of different animals upon exposure

(1941) and by Kamnev (1949). Our experiments were duplications of the experiments of Kamnev, with objective quantitative recording of the changes in translucency, and completely confirmed his results. We were, moreover, able to discover a phase of heightened translucency in the area of application of the anode.

Polarization was carried out for 1, 10, 30 and 60 minutes withcurrents from 0.015 to 4.0 ma. Curves constructed from 350 measurements were graphed in the same way as for muscles. Upon the application of a direct current of 0.015 ma., we were unable to detect any changes in translucency of the cornea in the area of the cathode (Fig. 3, D). Stronger currents invariably caused the appearance of the "cathode cataract" described by Kamnev and Prokof'yeva (1941), in which this occurred the more quickly the stronger the current. Just as in the case of muscles, we did not observe a phase of increased translucency at the beginning of polarization following the application of a cathode. However, this phase could be observed in the course of restoration of the original translucency after discontinuation of the current. The differences between the threshold strengths of currents capable of causing changes in translucency of the cornea under anode and cathode were greater than in the case of muscles. Changes in translucency under the anode were noted with currents exceeding 0.11 ma (Fig. 3, E). With weak currents (0.12 to 0.25 ma), these are observed as small but statistically reliable increases in translucency of the cornea under the anode. With further increases in current strength, a biphasic reaction was observed: an initial increase in translucency of the cornea was superseded by turbidity of it. Current strengths of 2.0 ma. and over caused turbidity immediately. A phase of increased translucency is observed in the course of restoration of the original transparency of the cornea after the development of "anode cataract". An increase in transparency is much less evident and the turbidity much greater in the cornea than in muscles. Experiments on restoration of the original transparency with the aid of anode polarization of a cornea rendered turbid following application of the cathode also yielded results comparable to those obtained with muscle. It is interesting to note that the intensity of "anode cataract" may be reduced with subsequent application of a weak current in the same direction.

Changes in transparency observed after application of direct current to tissues are apparently a substantive expression of so-called physiologic electrotonia. The restoration of transparency described by Kamnev and confirmed

in our experiments, which takes place in turbid tissues following application of the anode, correspond, it would seem, to the anelectrotonic restoration of the functions of altered tissues studied in detail by L. L. Vasil'yev and associates (Vasil'yev, 1937a, b., 1946, and others). Biphasic changes in translucency in the area of application of the anode may be compared with the evolution of anelectrotonia, and the nature of cathode and of secondary anode depression have their reflection in the reduction of anode turbidity following weak anode polarization.

Hence, translucency changes in a biphasic manner upon the development of parabiotic changes. The reduction of a light beam upon passing through biologic objects, especially in the case of colorless tissues, is primarily due to scattering of the light. Changes in light scattering in turn are connected primarily with changes in the degree of dispersion and orientation of submicroscopic particles of the cytoplasm. In addition, the optical properties are influenced by the interrelations of osmotic pressure and the indices of refraction of both the tissues and the surrounding medium. In the case of muscles, the relation between the magnitude of isotropic and anisotropic discs is also of importance. However, according to the findings of R. G. Lyudkovskaya (1949), changes of this interrelationship occur only in tetanic contraction. Therefore, findings concerning changes in light transmission, upon alteration under certain conditions (for example, upon exposure to non-electrolytes), enable us to judge the changes in dispersion, and permit an approximate assessment of their magnitude as compared with the original level. Starting from this, it may be assumed that, in a number of cases, development of the parabiotic process is connected with biphasic changes in the degree of dispersion of cytoplasmic colloids: an original increase with subsequent decrease. Increase in light transmission corresponds in time with reduction in the absorption of neutral red and with increase in the degree of polarization of the muscle, whereas turbidity corresponds in time with an increase in abdorption properties and a reduction in polarization (Kusakina, 1957).

It was of interest to compare the changes which we observed in light transmission with the optical changes of muscles upon stimulation. Muralt (1934) established that, with induction of tetanic contractions in muscles, there is usually a reduction in the coefficient of extinction. Very detailed studies of the optical properties of muscles in the process of isolated contraction were carried out by Schaefer and Gopfert (1937), who were able to show that, under certain conditions of recording, stimulation of muscles is associated with initial reduction and subsequent increase

in light scattering. Phasic changes in the transparency of muscles (increase at the time of initial relaxation and turbidity upon contraction) have been described also in the works of Hill (1948, 1949). The coincidence of changes in optical properties upon alteration and upon stimulation of muscle tissues is apparently not purely due to change. If this be so, then such coincidence once again confirms the notion of the common nature of local and spreading stimulation. (Vvedenskiy, 1901; Nasonov, 1959).

In conclusion I wish to express my gratitude to my supervisor, Leonid Leonidovich Vasil'yev for his constant interest and assistance during the execution of this work, and to Agripina Andreyevna Dobashina for her help in setting up the experiments.

Conclusions

1. Changes in the course of the parabiotic process in light transmission of the dermosternal muscle of frogs caused by compression, caffeine, chloral hydrate, ethyl alcohol, potassium chloride, calcium chloride, and the administration of a direct current, were detected with the aid of a photomultiplier.

2. With the development of the parabiotic process, light transmission changes in biphasic fashion: an initial increase in translucency is superseded by turbidity of the muscles. These changes are reversible, and an increase in light transmission may be observed after discontinuation of exposure to the altering influence.

3. Biphasic changes in translucency are seen best upon alteration with solutions of non-electrolytes and with compression. In these cases, the changes are apparently connected with biphasic changes in the degree of dispersion of the ctyoplasmic colloids.

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The Daily Periodicity of Mitoses in the Meristems of Barley

V. G. Grif

The Laboratory of Cytology of the Botanical Institute of the Academy of Sciences USSR, Leningrad

The periodicity of processes of growth in plants during the course of the day is a phenomenon which is well known in the botanical literature. Considerably less study has been given to the periodicity of mitotic activity in plants during the course of the day, although the first work devoted to a study of this problem appeared at the beginning of the present century (Lewis, 1901; Kelicott, 1904). Studies of the periodicity of cell division were made primarily on the meristems of root tips (Friesner, 1920, Ono, 1937, Movsesyan, 1951; Hageman, 1956), and only a few works were devoted to a study of the periodicity in the meristems of the vegetative cones of the stalks (Karsten, 1915; Rotta, 1948).

Despite the fact that there are a number of works on the experimental study of the causes of periodicity, there is no unified point of view on this problem. Certain authors believe that the periodicity of mitotic activity is an endogenous process, which is independent of external conditions (Friesner, 1920). Others point to a direct dependence between the periodicity of mitotic activity and the changes of external conditions during the course of a day (Lewis, 1901; Karsten, 1918). A number of authors conducting studies basically on roots or sprouts did not in general observe any marked periodicity (Winter, 1929; Rotta, 1949; Hageman, 1956), which must probably be explained by the type of nutrition proper to growing seeds. Growing seeds which receive nutritive substances from the endosperm or from the cotyledons show little dependance on variations in temperature and illumination, whereas the adult organism, having reached an independant stage of nutrition, depends entirely on external conditions, primarily on changes in illumination.

There is also controversy concerning the time of maximum and minimum mitotic activity, and there are no simultaneous studies whatsoever of the periodicity of mitotic activity in the meristems of root tips and the vegetative cones of stalks.

The present work is devoted to a simultaneous study of the periodicity of mitoses in the meristems of the vegetative cones of roots and stalks. This study is concerned only with the intensity of cell division and not with the increase in size of roots and stalks, since it is well known that during the periods of maximum intensity of cell divi-

sion, geometric growth (that is, the increase in size of organs) is minimal, and vice versa (Kelicott, 1904, Friesner, 1920).

TABLE

The Periodicity of Mitotic Activity in the Meristems of the Vegetative Cones of Barley in the Course of the Day

During the phase of the second leaf							During the phase of the fourth leaf						
	In the root			In the stalk				In the root			In the stalk		
The time of fixation	No of cells counted	No of dividing cells (in %)	$\frac{M_1 - M_2}{\sqrt{m_1^2 + m_2^2}}$	No of cells counted	No of dividing cells (in %)	$\frac{M_1 - M_2}{\sqrt{m_1^2 + m_2^2}}$	The time of fixation	No of cells counted	No of dividing cells	$\frac{M_1 - M_2}{\sqrt{m_1^2 + m_2^2}}$	No of cells counted	No of dividing cells	$\frac{M_1 - M_2}{\sqrt{m_1^2 + m_2^2}}$
9[00]	3200	19.4	—	1594	10.9	2.5	9[00]	4954	16.5	3.6	2197	14.9	4.4
12[00]	3700	18.8	3.4	1675	13.2	3.6	12[00]	5031	19.7	6.4	2146	19.4	2.9
15[00]	3752	28.8	2.4	2096	16.6	3.2	15[00]	4751	24.5	6.7	2034	21.5	[illegible]
18[00]	4112	33.6	2.8	1768	19.4	3.3	18[00]	5119	33.2	4.6	2105	21.2	[illegible]
21[00]	4098	28.2	2.7	2084	16.5	4.6	21[00]	5071	27.0	3.4	2084	26.0	3.4
24[00]	4002	23.3	3.0	2108	10.6	—	24[00]	4967	21.7	3.6	2028	17.7	1.5
3[00]	4421	18.5	10.6	1955	9.2	3.6	3[00]	5008	15.8	6.8	2124	14.5	3.1
6[00]	4452	7.2	11.4	2083	4.5	11.2	6[00]	5280	9.8	3.9	2066	11.1	[illegible]
9[00]	4897	19.0	—	2248	11.1	3.0	9[00]	5337	13.4	7.5	2012	15.2	3.5
12[00]	4893	19.3	4.6	2100	12.7	7.3	12[00]	5226	19.9	9.4	2163	19.9	2.9
15[00]	4783	30.0		2056	17.5		15[00]	5503	27.5		2313	25.7	

Methods

For the purposes of this study we used barley of the "Chervonets" variety, the seeds of which were obtained from the All-Union Institute of Plant Cultivation. Planting in open ground was carried out at two different times, at an interval of seven days. Fixation of the material for both times of seeding was carried out on the 7th and 8th of June 1956: for the first date of seeding, ten days after the appearance of sprouts during the phase of the fourth leaf; for the second date of seeding, four days after the appearance of sprouts in the phase of the second leaf. Fixations were made periodically over a course of 30 hours at intervals of three hours. Fixation of meristems of vegetative cones of roots and stalks was carried out simultaneously.

Each periodic fixation required 10 to 16 minutes. The material was fixed as described by Navashina (10:4:1). At the same time we recorded the temperature of the air (at the level of the soil) and of the soil at a depth of ten to twelve cm. (Fig. 1). Night time, during the time when we were making fixations, lasted from 10:30 PM until 3:30 in the morning.

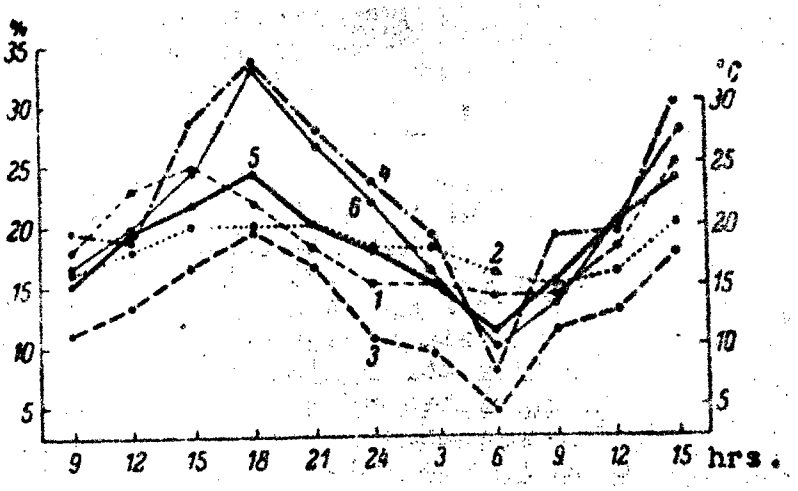

Fig. 1. Curves of Intensity of Cell Division in the Growth Cones of the Stalk and Root, and of the Temperatures of Air and Soil at Different Times of the Day.

Along the abscissa -- time of day; along the ordinate -- for curves 1 and 2, temperature; for the remaining curves -- intensity of cell dision (in percent of the total number of cells). 1 -- temperature of the air at ground level, 2 -- temperature of the soil at a depth of 10 to 12 cm., 3 -- intensity of cell division of the stalk during the phase of the second leaf, 4 -- intensity of cell division of the root during the phase of the second leaf, 5 -- intensity of cell division of the stalk during the phase of the fourth leaf, 6 -- intensity of cell division of the root during the phase of the fourth leaf.

From our fixed material, we prepared regular preparations according to the usual cytologic methods. Sections of 12 microns thickness were stained with iron hematoxylin by the Heidenhain method or by the Feulgen method, with a light green counter-stain.

Studies of the preparations were made with the aid of a tracing apparatus under strictly reproduced conditions. In each fixation, counts were made of the total number of cells and of the number of mitoses per microscopic field in

five central sections of five roots and of five vegetative cones of stalks. Counts were made in identical parts of these organs (Fig. 2, a to c). In calculating mitoses in the vegetative cones of stalks, we used a great coefficient, since the vegetative cone in the stalk is considerably smaller than in the root.

Our results can be seen in the table, which gives data on the number of dividing cells at different times of the day in percentages of the number of cells counted in five plants. This number of cells is taken as 100 percent, since the number of cells calculated varies in different fixations.

Results

As the result of time studies of the intensity of cell division in the meristems of vegetative cones of roots and stalks, we discovered that under normal conditions of growth there is a marked periodicity in cell division in barley.

Maximum and minimum numbers of dividing dells in the meristems of the root and stalk recurred at one and the same time. As can be seen from the table, the maximum percent of dividing cells in the meristem of the vegetative cone of a root occurred at 6 PM, reaching 33 percent, and the minimum at 6 AM, reaching 8 to 10 percent. In the vegetative cone of the stalk the times of maximum and minimum mitotic activity were the same (6 PM and 6 AM, respectively), but the variations in the percent of dividing cells were somewhat less: in plants during the phase of the second leaf there were variations from 19 to 4 percent, and in plants in the phase of the fourth leaf, variations by between 24 and 11 percent.

Consequently, the periods of increase and decrease in activity of cell division in different organs of the plants coincide. Moreover, even with changes in the intensity of cell division in the vegetative cones of stalks in connection with the transition of a plant into a succeeding phase of growth, the times of maximum and minimum cell division remained the same.

Consequently, the periods of increase and decrease in activity of cell division in different organs of the plants coincide. Moreover, even with changes in the intensity of cell division in the vegetative cones of stalks in connection with the transition of a plant into a succeeding phase of growth, the time of maximum and minimum cell division remained the same.

A more intensive cell division in the vegetative cones of stalks during the phase of the fourth leaf, as compared with the phase of the second leaf, was seen in all fixations. With changes in age of the plants, upon transition of plants from the vegetative phase of growth (Figure 2a) into the gen-

erative phase of growth (Figure 2b), the percentage of dividing cells in the vegetative cones of the stalk increased by an average of 5.5 percent. This increase was statistically reliable. In the vegetative cones of the root, no such change in the intensity of cell division was observed.

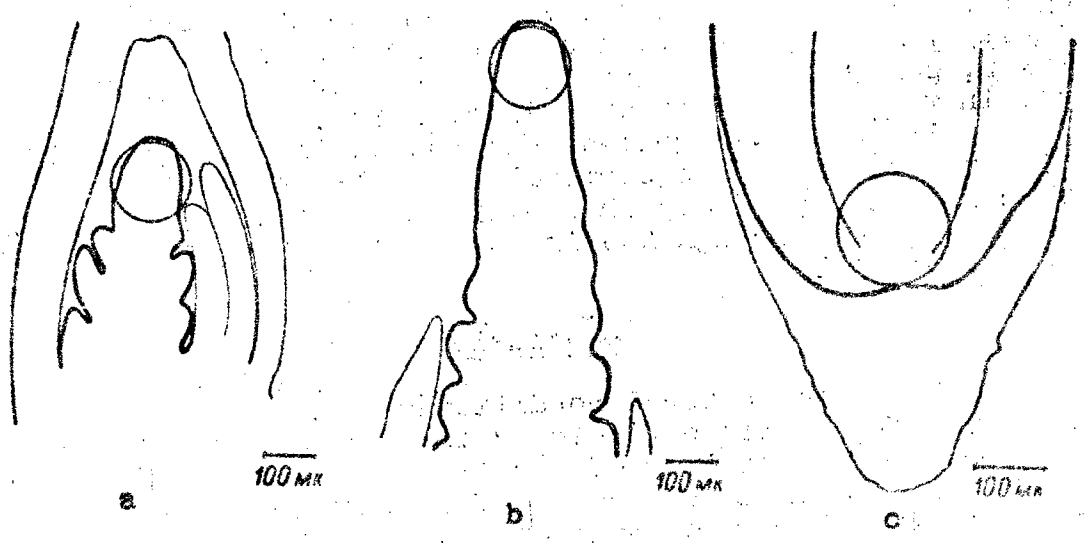

Fig. 2. Vegetative Cones of Stalks in Phases of the Second (a) and Fourth (b) Leaves and of the Root (c) in Barley (Schematic Representation).

Circles indicate those parts of the cones in which counts of cells were made.

As can be seen from the findings, we observed only one maximum and one minimum in the course of a day; however, in the majority of works studying the periodicity of cell division, there is evidence of the presence of two maxima and two minima in the course of twenty-four hours. The presence of only one maximum in our case may probably be explained by the considerable duration of the light period of the day (darkness from 10:30 PM to 3:30 AM, that is, only five hours).

As is known, the times of maximum and minimum cell division are different in different plants, but there is no definite moment when the number of dividing cells in maximum or minimum: each species probably possesses its own rhythm, which, moreover, may change with changes in external conditions. Rises and falls in cellular division do not depend on variations in temperature, since it is obvious from Fig. 1 that an increase in the number of mitoses comes on earlier than does a rise in temperature: the number of mitoses

begins to increase at 6 AM, whereas an increase in the temperature of the air does not begin until 9 o'clock, and not until later at a depth of 10 to 12 cm. in the soil. Moreover, as can be seen from the table and the graph, the range of variation of the number of mitoses in the roots is considerably greater than in the meristem of the vegetative cone of the stalk, although variations in temperature of the soil at a depth of 10 to 12 cm. were considerably less than the variations in temperature of the air and did not exceed 5°C. Hence, it is obvious that temperature is not a decisive factor in the periodicity of cell division.

It may be suggested that the periodicity of cell division depends on the periodicity of light conditions, although this dependancy obviously is not a direct one but an indirect one: through the influence of light on the metabolism of plants.

Conclusions

1. Under natural conditions of growth, barley exhibits a marked daily periodicity in cell division with one maximum and one minimum of division.

2. In the meristems of the stalks and the roots, maximum and minimum numbers of mitoses are seen at one and the same time. Consequently, the periods of rise and fall in activity of cell division coincide in different parts of the plant.

3. With changes in the age of the plant, the intensity of cell division changes in the vegetative cones of the stalk but does not change in the meristem of the root.

4. Even with changes in intensity of cell division in the vegetative cones of the stalk in connection with transition of the plant into a subsequent phase of growth, the dates of maximum and minimum cell division remain the same.

5. Temperature is not a decisive factor in the periodicity of mitoses in plants.

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The Effects of Different Concentrations of Ethylenediamine Tetra-Acetic Acid on the Microspores of Tradescantia Palludosa

N. L. Delone

The Laboratory of Radiation Genetics of the Institute of Biophysics of the Academy of Sciences USSR, Moscow

It has recently been shown in a number of works that, following exposure of living cells to ethylenediamine tetra-acetic acid, there are reorganizations of the chromosomes (Mazia, 1954; Levine, 1955; Wolff and Luippold, 1956; Kaufmann and MacDonald, 1957; Sax, 1957). The mechanism of action of this complex consists in the binding of bivalent cations, calcium and magnesium, in highly stable chelated compounds. Mazia (1954) stated that ethylenediamine tetra-acetic acid acts specifically on chromosomes, extracting calcium and magnesium from them. Starting with the fact that ethylenediamine tetra-acetic acid (EDTA) causes fragmentation of chromosomes, he developed the following scheme of the structure of chromosomes: blocks of nucleoproteins interconnected with calcium and magnesium ions. However, the opinion exists (Kaufmann and MacDonald, 1957) that EDTA does not act specifically on chromosomes but causes disruption of the general metabolism in the cell, due to which changes may occur in the cell nucleus. There is evidence that calcium deficiency in the cells leads to a reorganization of chromosomes (Steffensen, 1955). Hence, one of the causes of the development of spontaneous reorganization may be a disturbance of ionic balance in the cell. In connection with the fact that artificial extraction of calcium and magnesium ions also leads to chromosomal reorganization, the study of the morphologic features of chromosomal reorganization occurring in these experiments was of particular interest to us. We undertook to study the nature of the effect of EDTA on the cell nucleus in a very highly suitable object, the microspores of Tradescantia. Our findings give us some additional material for understanding both the role of ionic bonds in the structure of the chromosome as well as the role of deficiency of individual elements in the development of hereditary changes.

In our experiments, we used EDTA on the microspores of a single species of Tradescantia. The first post-meiotic mitosis in the microspores of this species of Tradescantia Palludosa requires a long period of time. Our experiments were carried out at temperatures of 19 to 21° C. At this temperature the first post-meiotic mitosis lasts for ten

days. Cells of the anthers in a single flower bud mature relatively synchronously, with a delay of certain individual cells by as much as 12 hours. In inflorescence, the buds develop non-simultaneously: at the time when the upper blossoms inflioresce, the anthers contain binuclear pollen; in the lower buds the cells of the anthers have not yet entered meiosis.

The method of work was as follows: With a syringe a solution of the acid was injected in all the buds of a given head simultaneously. Then fixations were made of the anthers from buds in which microspores had reached the metaphase of the first post-meiotic mitosis. Due to the fact that the duration of each phase of mitosis during the development of microspores had been established earlier by us, and with our knowledge of the moment of exposure and of the moment of fixation, we were able to determine, in each case, the phase of the mitotic cycle at which the cells had been subjected to the acid. All evaluations of reorganization of the chromosomes were carried out in metaphase. The haploid number of chromosomes in Tradescantia palludosa is six. The chromosomes of large dimensions, which differ very little in size, all possess a submedial attachment of the spindle in which the centromere of three of the chromosomes lies somewhat closer to the middle than that of the other three (see Figure). We worked with plants of the Sax clone, in the microspores of which spontaneous reorganizations of the chromosomes do not occur.

Metaphase of the First Post-Meiotic Mitosis of Tradescantia Palludosa (microphotograph, magnification 1300).

a -- normal; b -- central chromosome ring and fragment arising upon irradiation with X-rays (200 r) of cells in early prophase; c -- chromosome fragments arising upon exposure to EDTA for 42 hours, the initial exposure having occurred in the early prophase.

EDTA was used in concentrations of 0.05 M to 0.001 M, in which the pH of the solution in all experiments was 7.0. The time of exposure in the different experiments was

from 1 to 24 hours. However, the time of exposure in our experiments was somewhat arbitrary due to the peculiarities of the method used. The solutions were admitted in a continuous stream over a period of five minutes every half hour for a day, with an interruption of 10 hours at night.

Fixation of the anthers was carried out in a mixture of absolute alcohol and glacial acetic acid (ratio of three to one) for a period of fifteen minutes; then they were washed in 70 percent alcohol and acetocarmine preparations were made.

Depending on the concentration of EDTA and the time of exposure, we observed varying degrees of injury of the cells: complete death of cells, absence of entry of cells into mitosis, confluence of chromosomes in prophase and metaphase, reorganization of chromosomes, and slight postponement in the time of occurrence of different phases of mitosis. It should be kept in mind that cells, as they go through the cell cycle, may possess different sensitivities to certain influences. Thus, a number of authors (Sax and Swanson, 1941; Bozeman and Metz, 1949; Bishop, 1950; Deschner and Sparrow, 1955) have established that, upon exposure to ionizing radiation, the most sensitive cells are cells in metaphase and the least sensitive are those in interphase, if the criterion of sensitivity is the number of reorganizations of chromosomes.

Complete death of cells in our experiments occurred when they were exposed in any phase of the mitotic cycle to a solution of EDTA in concentrations of 0.05 M to 0.01 M for one hour or more. The cytoplasm was dried out and the cell membrane deformed.

Exposure to a solution of EDTA in a concentration of 0.06 M for three hours led to failure of entry of cells into mitosis if they were in interphase at the time of exposure. Pollen formation was abortive.

Confluence of the chromosomes was seen with concentrations of EDTA of 0.002 M after exposure times of 24 hours, if the exposure was started in the middle or later prophase. The matrix of the chromosomes became confluent in a number of places, and upon transition to anaphase, unique bridges were formed between the chromosomes located towards the poles.

Reorganizations of chromosomes occurred upon exposure to EDTA in concentrations of 0.001 M for 42 hours, when exposure was started in early prophase. In this case, the chromosomes lost a part of their matrix and their spiral structure could easily be made out. The figure represents such a cell with a chromosome fragment (c). For comparison, photographs of two other cells are shown. Normal metaphase (a) and a cell with a central chromosome ring, along with a fragment after X-irradiation of it in early prophase (b), are

shown. As can be seen, the condition of the matrix upon exposure to EDTA is markedly changed, whereas X-rays do not cause any changes in the matrix as compared with the normal.

In experiments on the effects of EDTA in concentrations of 0.001 M (42 hours) on cells in early prophase, we counted 210 cells, that is, 1260 chromosomes. The total number of reorganizations was 1.11 ± 0.29 percent. The ratio of fragments (0.79 ± 0.25 percent) to dicentrics (0.32 ± 0.16 percent) was 2.5. We noted both chromosomal and chromatid reorganizations. The ratio of the sum of chromosomal and isochromatid reorganizations to chromatid reorganizations was 2.5.

We exposed cells in interphase to an 0.001 M solution of EDTA for a period of 42 hours. In this experiment we were unable to detect any reorganization of chromosomes; on the other hand, we counted altogether only 480 chromosomes. The matrix of the chromosomes stained slightly less well with carmine than did the normal.

Hence, with high concentrations of EDTA, there is complete death of cells, but it is possible to arrange such concentrations and exposure times as will lead to fragmentation of chromosomes by EDTA. It is possible that EDTA changes the concentration of ions throughout the cytoplasm of a cell, due to which there may be disruption of ionic bonds within the chromosomes, which leads to the development of reorganization in it.

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From the History of Science

(The History of the Discovery of Karyokinesis)

Z. S. Katsnel'son

In the development of cytology, the discovery of karyokinesis and the clarification of the nature of mitotic division constitutes one of the most important triumphs. Therefore, the history of this discovery is naturally of interest. However, both in the foreign and in the Soviet literature, the reader may find highly conflicting information on this question.

Here are some examples. G. Petersen (1922) wrote, "The true discoverer of the mitotic figure of cell division was Butschli (1876). Later this phenomenon was studied in detail (Flemming, in the 1880's; Stasburger, studying the plant cell; van Beneden, K. Rabl', and Boveri)" (Page 88). Another example isthe work of V. Bargmann (1948). In the textbook on cytology by Ries and Gersch (1953), a table is given of the basic cytologic discoveries in which we read, "In 1844, K. Nageli first observed mitotic figures in plant cells," and "in 1849, V. Hofmeyster described the course of mitosis." "In 1867," the authors further state, "V. Hofmeyster described indirect division or karyokinesis in great detail in plant cells" (Page 495). The overwhelming majority of foreign authors (Hertwig, 1912; Kuster, 1938, and others) exhibit a characteristic ignorance of the works of our Soviet investigators who have studied cell division.

P. I. Peremezhko (1887), a Russian histologist who was a direct participant in the discovery of karyokinesis, stated at the end of the nineteenth century, "The indirect division of cells was observed for the first time simultaneously in living animals by Flemming in the larvae of salamanders, by Peremezhko in the larvae of the newt, and by Schleicher in amphibia and mammals, describing the process in considerable detail" (Page 74). A. V. Nemilov (1941) believes that "before the end of the 1870's the details of this process (of mitosis) had not yet been clarified, and it was not until 1878 that a Russian scientist, Peremezhko, and a Russian histologist, Flemming, gave the first coherent descriptions of this process (Page 158). In the textbook of A. A. Zavarzin and S. I. Shchelkunov (1954), it is pointed out that "Mitosis was first described in plant cells by I. D. Chistyakov in 1874. In animal cells it was studied first by Peremezhko in 1879" (Page 87). The article of Ye. A. Shchubnikova (1952) does not afford an objective appraisal, although it is a special historical article. In her opinion, "The

study of original works has shown that the true discoverers of indirect division were Chistyakov, Mayzel', and Peremezhko," (Page 380). In the author's monograph devoted to the history of the study of cells (Katsnel'son, 1939), the history of the discovery of karyokinesis is set forth with insufficient completeness and precision. Comparatively recently, the serious study of the English cytologist, Baker, was published (1955). In it, an account is given of the works of our Soviet authors, but their significance is insufficiently appreciated.

All this has compelled the present author to give in this article a brief essay on the history of the discovery of karyokinesis based on a study and comparison of original works relating to the problem at hand.

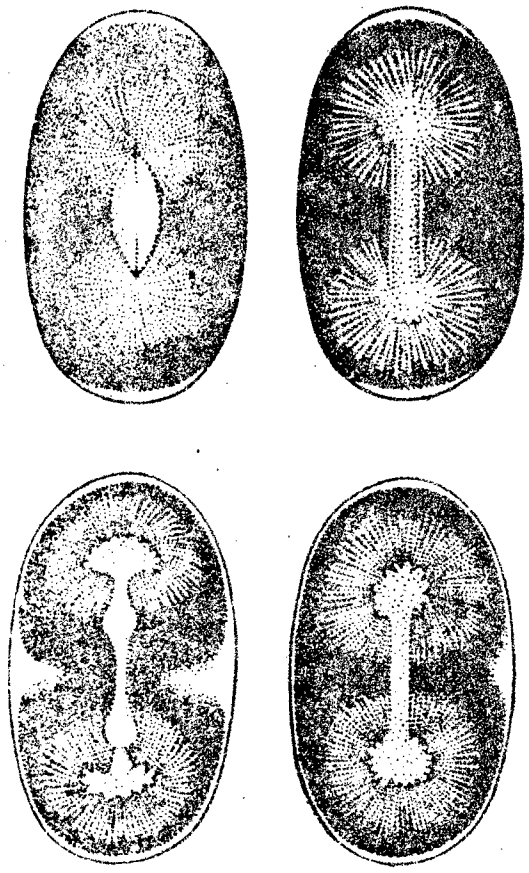

Fig. 1. "Karyolysis" According to Auerbach (1874). Explanation in the text.

When the name of one or another investigator is mentioned and the "discovery" of kayokinesis is attributed to him, a false impression is created in the mind of the

contemporary reader. Indeed, we now know that karyokinesis is a complex process connected with a number of transformations in the cell. The sum of our knowledge concerning mitotic division was accumulated gradually and the authors who first "described" karyokinesis, or, as it is usually expressed, "discovered" it, in actuality had a very unclear notion of the essence of this process and of the importance of the complex morphologic picture which they observed.

The works of Mol', Zheleznov, Nageli, Remak, Virchow, and a number of other investigators have made clear the incorrectness of the theory of cytoblastemes of Schleyden and Schwann, and have securely confirmed the idea of the continuous growth of cells by means of division (see Katsnel'son, 1939; Baker, 1953). Remak developed the scheme according to which there is first division of the nucleolus, then constriction and division of the nucleus, and finally division of the cell. However, beginning with the middle of the nineteenth century, many investigators noted that, at a definite stage of division, the nucleus in the cell disappears and then reappears anew in the daughter cell. In order to designate this phenomenon, a special term was created -- "karyolysis." During the period of dissolving of the nucleus, investigators sometimes observed certain ray-like formations in the cytoplasm. The pictures of cell division in a number of works of the 1860's and 1870's are very characteristic. An idea of them is given in Fig. 1, which is taken from the work of Auerbach (1874). Auerbach was convinced that the nucleus of the maternal cell completely dissolved morphologically and disappeared, and was restored anew in the daughter cells. Some investigators, for a long time, observed either these or other elements of the mitotic figure, and by chance described or even drew figures of separate stages in division, but did not attribute to them any importance and did not see in them a special form of cell division. Thus, K. M. Ber (1947), in describing experiments on artificial fertilization in sea urchins, observed changes in the fertilized egg. From his descriptions, it can be ascertained that he saw the silhouette of a mitotic figure. Ber does not give a drawing, and of course, at that time, he did not understand the significance of the picture which he saw.

Some authors believe that Nageli (1942) was the first to see chromosomes (Baker, 1955). This opinion is based on one of the figures in the work of Nageli, in which he represents, in Lilium tigridum, the process of disappearance of the "cytoblasts" of the maternal cells and tha appearance in their place of varying numbers of smaller cytoblasts of a transitory character. In his drawing, Nageli shows some large spheres to which there is no reason to attribute a

chromosomal nature. Therefore, the opinion of Ries and Gersch cannot be regarded as well-founded when they suppose that Nageli was the first to observe mitotic figures in plant cells.

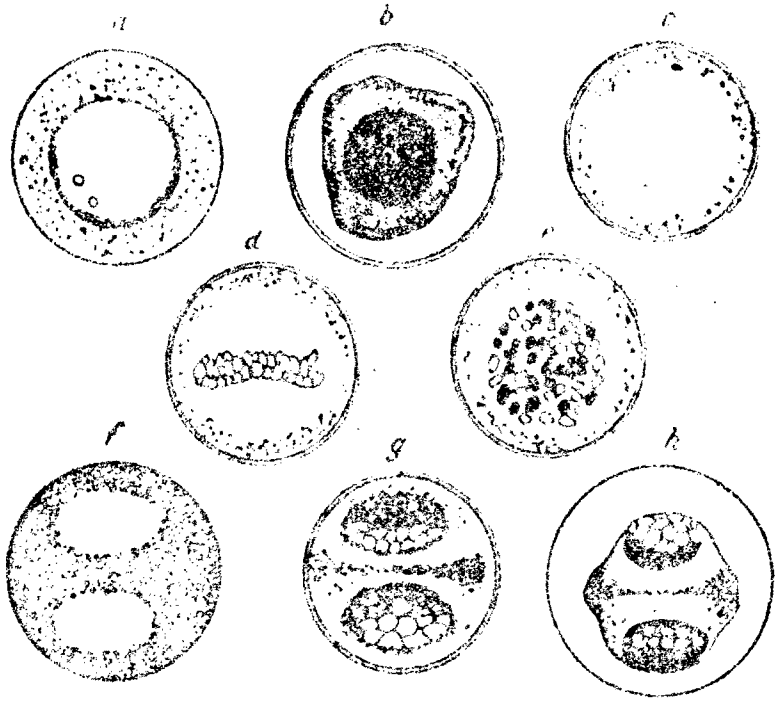

Fig. 2. Division of Spore Cells of Lycopodium psilotum triquetrum, According to Hofmeyster. (1867, Fig. 16)

Hofmeyster (1849), in his studies on the development of the embryo in the phanerogamia, represents in one of his drawings division of a cell in Tradiscantia, in which, instead of two nuclei, he shows groups of bodies which rather remotely resemble chromosomes. Hofmeyster, however, was inclined to attribute this unusual type of dividing cell to the action of water. In his much later "Study of the Plant Cell" (1867), Hofmeyster represents the sporulating maternal cell of Lycopodium psilotum triquetrum in various stages of division (Fig. 2). After showing the stage when the "first nucleus disappears" (in the figure the cell is completely empty -- in drawing c), Hofmeyster further indicates stages d and e in which, in the center, are shown angulated clumps remotely reminiscent of chromosomes. The caption to the figure reads "The same cell after a brief period in water. The proteinaceous liquid in the center has congealed in irregular clumps which are grouped about the equatorial disc of the cell in the form

of a plate" (Pages 82 to 83). Of course, such an observation cannot be regarded as the discovery of karyokinesis, and the statement made by Ries and Gersch, who believed that Hofmeyster described karyokinesis in plant cells "in detail" in 1867, is not actually applicable to him.

V. Krause (1870), in his work on corneal epithelium, makes a remark which superficially anticipates descriptions of the figure of division; he does not, however, give drawings.

One of the first to see perfectly clearly and to represent uniquely one of the figures of karyokinesis was A. O. Kovalevskiy in his "Embryologic Studies of Worms and Arthropods" (1871). He gives figures (in the original, this was Fig. 92, reproduced here in Fig. 3), which indisputably correspond to the picture of anaphase. This is a good representation of a mitotic figure with a spindle and chromosomes. However, Kovalevskiy, with respect to his observations, limited himself to the following brief remark: "The body of the nucleus stands out in the sections as a dividing vesicle, but there are seen, both in the old and in the newly-formed cells, two granular accumulations which are joined to each other by finely granular, but very clearly visible, protoplasmic (?) strands." (The question mark was inserted by Kovalevskiy.) The granular accumulations are, of course, chromosomes, and the strands are the central spindle. Hence, A. O. Kovalevskiy was one of the first to see and describe the basic elements of the mitotic figure. Unfortunately, this precise observation which he made remained outside his sphere of interest, and hence he did not assign to it any importance and did not develop it further.

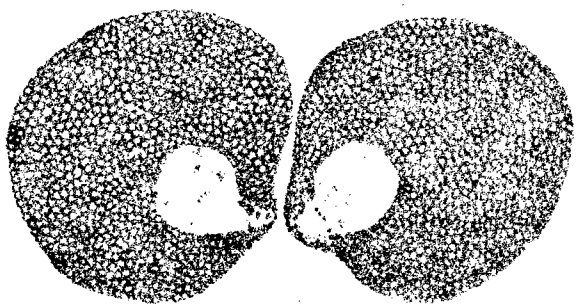

Fig. 3. Separation of Micromeres in Euaxes, According to A. O. Kovalevskiy (1871, Table 6, Fig. 24).

Edmund Russov, a teacher in Revel, and later Professor of Botany in Derpt (for more details concerning Russov, see Winkler, 1897), in describing the development of spores

In ferns (1872), represents, in a number of his figures, cells in which he saw sheets of granules or fine bands during division which were undoubtedly poorly preserved chromosomes. In distinction from Hofmeyster, Russov declined to regard these structures as artefacts and understood that the pictures observed by him reflected certain peculiarities in division, but the importance and significance of this feature remained unclear for Russov.

It is astonishing that the first and, for that time, very good description of karyokinesis passed unnoticed. It was made by the Hessian zoologist, Schneider (1873) (for a biography of Schneider, see Limpricht, 1890) in 1873. In the turbellarian Mesostomum, Schneider described and well illustrated karyokinesis in dividing eggs; metaphase is very clearly shown (both from the equator and from the poles), and also the splitting of chromosomes, which the author refers to as "heavy loops." From the description and the figure, no doubt remains that Schneider saw actual mitosis (Fig. 4). (A description of karyokinesis is given in the work of Schneider on pages 114 to 116, and the figures are reproduced in Table 5. Ye. A. Shchubnikova (1952) affirms that Schneider was unable to see the formation of chromosomes and their splitting. The extent to which this is incorrect is readily seen from the reproduction here of his figure.) He observed similar pictures of division also in other turbellarians, and states that he saw comparable pictures of division in some cells of the intestinal tract. In actuality, this was the discovery of karyokinesis. But Schneider described his observations in a long zoologic study entitled "Studies on the Flat Worms," in which the discovery of indirect division of cells was embedded in a mass of other factual material; furthermore, this work was printed in a poorly distributed publication of a local scientific society.

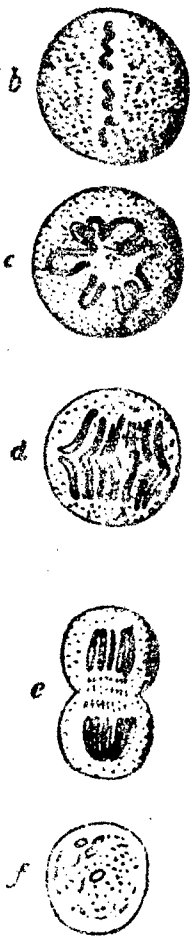

Fig. 4. Division of the Egg in Mesostonum ehrenbergii, According to A. Schneider (1873, Table 5, Fig. 5).

Schneider himself, however, evidently understood that he had witnessed a special form of division of cells, but considered this form a peculiarity of the flatworms. Be this as it may, this remarkable discovery did not attract attention, and the fact that karyokinesis had been described in the article by Schneider received public attention only when the investigations of other authors attracted general attention to mitotic division.

The work of Schneider concludes the first preparatory period of the discovery of karyokinesis. In the ensuing period, over the course of several years, a whole series of studies appeared which showed that investigators were dealing with a hitherto unknown method of cell division widely distributed both in plants and animals.

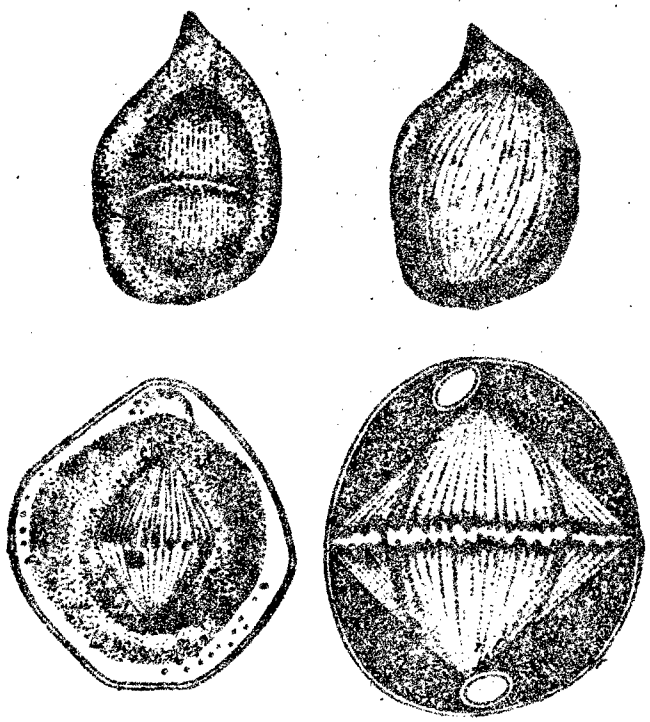

Fig. 5. Drawings of Cells Dividing by Karyokinesis, According to I. D. Chistyakov (1875, Fig. 9, 24, 20).

This new period began with the works of the Russian botanist, I. D. Chistyakov (for a biography of I. D. Chistyakov, see S. S. Stankov, 1946). The first indication of a special form of cell division is given in the work of I. D. Chistyakov entitled "Materials on the History of the Plant Cell" (an abstract of this work concerning indirect division is given in a Russian translation in the collection

"Russian Classics in the Morphology of Plants," 1923) and printed in an Italian botanical journal in 1874. In the following year, the article appeared in a German botanical journal and was entitled "Materials on the Physiology of the Plant Cell" (Chistyakov, 1875). In these works, I. D. chistyakov describes his observations on the growth of spores and anthers, which he studied in living material exposed only to the action of water. Chistyakov distinguishes a multiplicity of phases, and his terminology is quite different from the modern terminology. Thus, the figure of the metaphase was called by him the "band nucleus," chromosomes were called "papillae." His study of unfixed and unstained material did not permit him clearly to discern the transformations of chromosomes, and therefore Chistyakov concentrated his attention primarily on changes in the spindle. He undoubtedly saw the maternal and daughter stars ("centrosome" in modern terminology) and represented them in his drawings (Fig. 5).

One cannot but be astonished at the observations of Chistyakov, who was able in living material to detect, in some cases very precisely, the changes associated with indirect division. In distinction from authors who held to the theory of karyolysis, I. D. Chistyakov understood the succession of maternal and daughter nuclei. In describing the formation of "secondary" (daughter) nuclei, Chistyakov wrote, "This process is thought of as the free formation of nuclei after dissolution of the first nuclei. However, in view of what we described earlier, it must be acknowledged that it is difficult to accept this position. We observed the division of the striated sphere; we followed the differentiation (or to express it better, the transformation) of different parts of this sphere, and now we are in a position to indicate precisely the parts of the sphere which give rise to the secondary nuclei; they derive from the half-spheres described in the preceding phase, and the latter, in their turn, are polar segments of the stage of the striated sphere. Hence, it is necessary to admit that nuclei persist during the whole course of division" (Page 20 of the Russian translation). Hence, I. D. Chistyakov understood the succession of nuclei in division of the cell, and pointed out that in this process there is formed a complex figure of division, but he was unable to follow the transformations of chromatin and supposed that the daughter nuclei are formed at the poles of the spindle from the threads of the sphere. There can be no doubt that only disease and premature death deprived this talented Russian scientist of the possibility of continuing his investigations and of perfecting and developing his first observations,

which the author himself called "preliminary generalizations" (foreign authors have hitherto passed over in silence the works of Chistyakov, Hus and Kuster (1938) in their essay on the development of our knowledge concerning the plant cell in which, generally speaking, they do not overlook Russian authors, do not even mention Chistyakov. Baker (1955) gives some attention to Chistyakov in his most recent collection.).

In 1875 appeared the first studies of Strasburger, who was a native of what was at that time Russia (Strasburger was born and began his scientific and pedagogic activity in Warsaw). This study was published in the form of a monograph under the title "Cell Formation and Cellular Division." In it he gives descriptions of karyokinetic division in a number of plant specimens and attempts to show that the new form of division is not a rarity but, on the contrary, is a typical form of cell division. For this purpose, Strasburger devotes attention to objects from the animal world. He attempts to study division in cartilaginous cells; he describes and gives representations of division of eggs in ascidians; and he uses the figures of Butschli (see below), who detected division in a nematode. Strasburger unquestionably advanced the study of mitosis greatly. He described more clearly the equatorial plate of chromosomes and its cleavage, but the chromosomes ("bands" or "ribbons") were regarded by Strasburger as a thickening of the threads of the spindle, believing that the daughter nuclei are formed by confluence of the "bands" with each other and with half of the spindle. In this work, Strasburger gives evidence of no understanding of the initial and final stages of mitoses as yet, and concentrates his entire attention on the stage currently designated metaphase and anaphase.

Strasburger takes up cell division again in a second monograph (1878), which is devoted for the most part to the phenomenon of fertilization. In addition to plant objects, he here concerns himself with the division of cells of a number of animal tissues also. In particular, Strasburger uses the preparations of the Warsaw histologist Mayzel' (see below), from which he takes a whole series of drawings (Fig. 6). Later, Strasburger repeatedly turned to the problem of cell division (1879, 1882, 1884). It cannot escape notice that in the works of Strasburger, there was a close tendency to ignore the studies of I. D. Chistyakov, or else to deride him, pointing out his mistakes and ignoring the important aspects of his observations. (On this matter, K. A. Timiryazev wrote, "It is necessary to take note of this, in view of the not altogether justified attitude towards Chistyakov of another Russian botanist of the same period. Some know that the now famous Professor Strasburger was, at the end of the 1860's, a Docent. In his recent

essay on the history of this problem, Strasburger does not give sufficient attention to Chistyakov, whereas he is the only one of the German botanists who is well acquainted with the contents and time of appearance of the works of Chistyakov and even knew him personally," K. A. Timiryazev, "Collected Works," Vol 8, Page 161.)

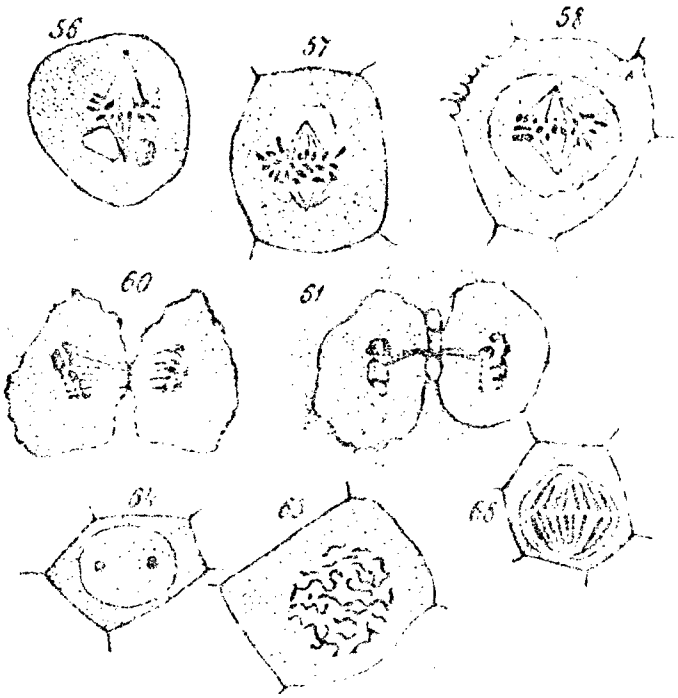

Fig. 6. Drawings of Cells of the Corneal Epithelium from Preparations of Z. I. Mayzel', from the Monograph of Strasburger (1878, Table 7).

In 1875, a preliminary report appeared on the division of eggs in the roundworms and in snails, published by Butschli (1875). The author briefly notes certain aspects of indirect division, without accompanying them by figures -- it was noted above that these were reproduced in part in the monograph of Strasburger (1875). In 1876 the monograph of Butschli came out under the title of "Studies of the First Stages of Development of Egg Cells, of Cell Division, and of Conjugation in the Infusoria." This was a large work, with generous use of tables and with descriptions of the primary stages of division of eggs in worms and molluscs, and of division of the spermatogenic cells of the cockroach, of the embryonic blood cells of the chick, of the leukocytes of amphibia, and of the blastodermal cells of insects. Butschli, like Strasburger, repeatedly emphasizes that the new form of division of cells is inherent alike both to plants and to animals. The general

level of description of mitosis in Butschli's book is approximately the same as in the first work of Strasburger.

In the same year (1875), the first report of V. I. Mayzel' appeared (Vyacheslav Iosifovich Mayzel' graduated in 1870 from Warsaw University and was appointed assistant in the department of histology under Professor G. F. Goyer. He worked in the department of histology until 1895, and when Goyer left the department, Mayzel' was recommended as his replacement; however, as a Pole, he was not acceptable to the Tsarist government, and leaving the University, he took up medical, scientific, and literary work. Regarding him, see "Gazeta Lekarska," Vol 27, No 2, 1907, Pages 44 to 48). In this article, which was entitled "Unique Manifestations in the Division of Nuclei in Epithelial Cells," Mayzel' reported that, in observing regeneration of corneal epithelium, he had long previously noticed in some cells "numerous large granules or fibrillar formations" (Mayzel', 1875A, Page 864). He had not previously been able to come to any conclusions on the significance of these formations, but, having become acquainted with the works of Strasburger and Butschli (published in the same year but somewhat earlier), Mayzel' reviewed his preparations and noted that the pictures observed by him completely corresponded to cell division as represented by the above-mentioned authors. Mayzel' describes in his article three forms of nuclei: the first is approximately that of prophase, but is not clearly described; the second belongs to metaphase, and the third to anaphase. These same findings are discussed in an article by Mayzel' published in the Polish language (1875B).

In a subsequent communication (1877), Mayzel' writes of two additional forms of nuclei which apparently belong to telophase. These findings are taken up in greater detail in an extensive study on the regeneration of epithelium published in 1878 in Russian in the "Works of the Warsaw University." Here, there is a special chapter entitled "Nuclear Division" in which Mayzel' describes in detail the five forms of nuclear structure associated with division. He does not refer to the process of division itself, but the very order in which Mayzel' describes the five nuclear forms corresponds to subsequent ideas of the course of karyokinesis (in this article he makes reference to numerous figures, but the article remains unpublished: it has been shown that the end of the work (and apparently the Tables of figures) was to be included in a subsequent issue. But in none of the succeeding issues (there were four in all) was there any continuation of Mayzel's article, and it was apparently never published at all. The drawings from the preparations of Mayzel' were published by Strasburger (1878), of whom we

have spoken earlier). In an article of 1879, Mayzel' reports his studies of ascarids, in which Auerbach (see above) described karyolysis. Mayzel' indicates that in the roundworms it is possible to see "typical fibrillar nuclear spindles with a granular equatorial plate and with fibrillar rays at the poles of the spindles" (Page 281). He saw similar pictures in the eggs of the slug and in the cells of the ectoderm. In 1884, Mayzel' published a long article in Polish on karyokinesis and supplied two tables of figures. (This work was published by Mayzel' in a collection in honor of Professor Goyer. Unfortunately, I was unable to find this book.)

At the same time, the work of Beneden appeared (1875), who studied cell division in the embryonic ectoderm of the rabbit after osmic fixation and staining with picrocarmine and hematoxylin. His data are less abundant than those of Mayzel'. Beneden saw cleavage of the equatorial plate into two discs and described the onset of anaphase. Later, Beneden studied division in Dicyemella (Beneden, 1876).

A similar approximate description of mitosis was given in the first work of Fol (1876). Later, in 1879, Fol published an extensive study devoted to maturation, fertilization, and the first stages of growth in non-vertebrates (achinoderms, malacozoans, and others). Here, he gives a clear representation of mitotic figures with formation of reduction bodies in the first stages of division. However, as was the case with the majority of authors of the preceding period, Fol concerns himself primarily with the middle phases of karyokinesis and gives no clear idea of the transformations of the equatorial plate.

A somewhat clearer description of the course of indirect division is given by Balbiani (1876). In the ovaries of an orthopteran (Stenobotrus), he found a suitable object for studying the figures of division. Balbiani does not give any drawings, but the general level of his descriptions remains within the limits of the conceptions characteristic of the initial descriptions of phases of karyokinesis.

Cell division in the cornea was considered in the work of the Zurich pathologist Eberth (1876). This author believes that a special feature of division of animal cells (as compared with plant cells) is the fact that, in the latter, a differentiation of the nuclear mass begins with the formation of an elongated striation, and that, in the corneal cells, there immediately appear "equatorial granules" (that is, chromatin), which "if not always together, then in any case for the most part, serve as material for the daughter nuclei" (Page 538). In the work of Eberth, an

essential place is given to the changes in chromatin; he clearly describes chromosomes and observes not only the middle phases of division but also the initial phases; in particular, he gives an excellent representation of prophase (Baker, 1955, believes that the first representation of prophase in the endothelial cells of Descemet's membrane is given in the works of Ewetsky (1875). I was not able to find the works of Ewetsky in the original).

The Kiev zoologist, N. V. Bobretskiy (1877), in a study of the embryonic development of the univalve molluscs, describes division in Nassa mutabilis, and clearly portrays the mitotic figures with central spindles, asters, and chromosomes. However, in criticizing certain incorrect ideas of Butschli, Bobretskiy himself gives an incorrect description of division. Thus, the onset of anaphase (Fig. 24 of the original) is described by him as a remnant of the maternal nucleus, which he understands as being shared between the daughter groups of chromosomes.

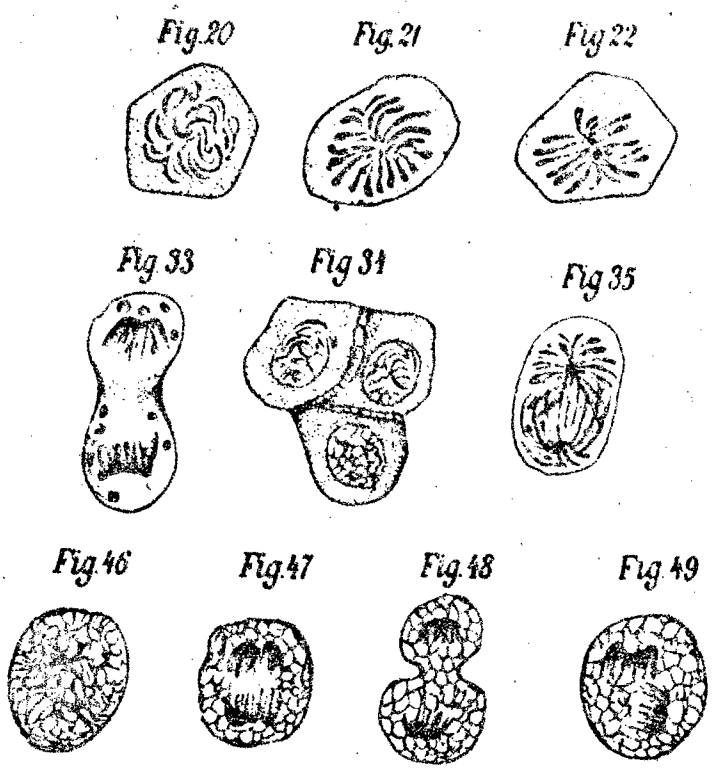

Fig. 7. Drawings of Dividing Cells of Larvae of the Newt, from the Article of P. I. Peremezhko (1879A, Table 19).

By the end of the 1870's, the new form of cell division was attracting the attention of an ever-increasing

number of histologists. Almost simultaneously there appeared the major works of three authors interested in indirect division: Peremezhko, Schleicher, and Flemming.

The first brief report of the Kiev histologist P. I. Peremezhko (concerning P. I. Peremezhko, see O. Chernyakhivs'kiy, 1928; and M. K. Kuz'min, 1957) was published in July of 1878. The author introduces a new object for studies of division of different cells, which is very suitable due to its transparency; this is the tail of the young newt. Using this object, Peremezhko observed cell division in the epidermis, connective tissues, endothelium, and leukocytes. In the succeeding year, the complete work of P. I. Peremezhko appeared (1879A) with tables and drawings. He had studied both living and fixed material, but had not used stains (Fig. 7). During this same year, Peremezhko published yet another study devoted to division of erythrocytes in amphibians (1879B), in which he gives in general a correct idea of the sequence of stages.

In the second part of his major work (1880) (the second part of the work of P. I. Peremezhko was published in the next volume of the same journal; it was devoted to a description of division in connective tissue cells and endothelial cells), P. I. Peremezhko, in contrast to the first work of Flemming which appeared at about the same time, was inclined to divide the entire process of division into two stages: the first stage begins with the onset of changes in the nucleus and lasts until the beginning of its division, and was called by Peremezhko "the stage of nuclear changes"; the second stage begins with the onset of nuclear division and ends with complete division of the cell, and was called by Peremezhko the "stage of nuclear or cellular division" (Page 184). P. I. Peremezhko believed firmly that the use of stains did not afford any additional advantages for histologic study. This position indisputably prevented his more active participation in working out the problem of karyokinesis.

Simultaneously with the works of Peremezhko, the studies of Schleicher appeared (1879), which were devoted to division in cartilaginous cells. His preliminary reports came out in the same journal which published the article of Peremezhko (several issues earlier, in June of 1878), and his complete work in the sixteenth volume of the "Archives of Microscopic Anatomy." Schleicher did not introduce any essentially new data, but we are indebted to him for the now commonly used designation of indirect division -- karyokinesis.

The third author whose work appeared during 1878 and 1879 was Flemming, who made a particularly important contribution to the study of karyokinesis. The first report of

Flemming was published in 1878 in the works of the scientific society in Kiel; this report appeared on the first of August 1878. Even in this brief report, Flemming gives an outline of cell division by mitosis which is clearer than that of the majority of his predecessors. Flemming attempted to follow all of the transformations of dividing cells from the state of the nucleus seen in a cell which is not dividing to complete reconstruction of the daughter nuclei. He devoted major attention to the stainable substance of the nucleus, and by using different methods of staining, attempted to follow its transformations more precisely. At the end of his report, Flemming remarks that, on the day of finishing his report, he had become familiar with the first preliminary report of Peremezhko. Flemming notes the many similarities in the figures which Peremezhko describes for the newt and which he observed in the salamander; the slight discrepancies are explained by Flemming by the fact that Peremezhko did not study stained preparations. In the same volume, the article of Flemming was published entitled "Remarks on the Structure and Life of Cells and on the Limits of Visibility." In this, Flemming also takes up the problem of mitotic division and presents a table of drawings which quite clearly, even though schematically, illustrates the course of karyokinesis.

In 1879, Flemming published in the Virchow "Archives" an article on "The Behavior of the Nucleus in Cell Division and the Significance of Multinucleate Cells." In this article, he introduces the designations "direct" and "indirect" division and criticizes the application of the Remak scheme of direct division to the majority of animal tissues. Flemming calls attention of pathologists to the necessity of revising the old ideas concerning cell division and gives, by way of illustration, a table in which he represents the course of mitosis. Here, Flemming clearly indicated the longitudinal splitting of chromosomes, which hitherto had not been seen clearly by other investigators.

In the same sixteenth volume of the "Archives of Microscopic Anatomy" in which the articles of Peremezhko and Schleicher had been published, there appeared the first part of the major work of Flemming entitled "Materials on Our Knowledge of Cells and Their Manifestations of Life Activity" (Flemming, 1879B). He presents here in detail the changes in the structure of the nucleus and the transformations of chromatin and of achromatin, and for the first time calls attention to the role of the centriole. The nature of the changes in chromatin and the formation of chromosomes is clearly represented here. Flemming called attention to one of the most important points in karyokinesis -- the longitudinal splitting of the chromosomes. The article of Flemming

was accompanied by superb illustrations, some of which are reproduced in Fig. 8.

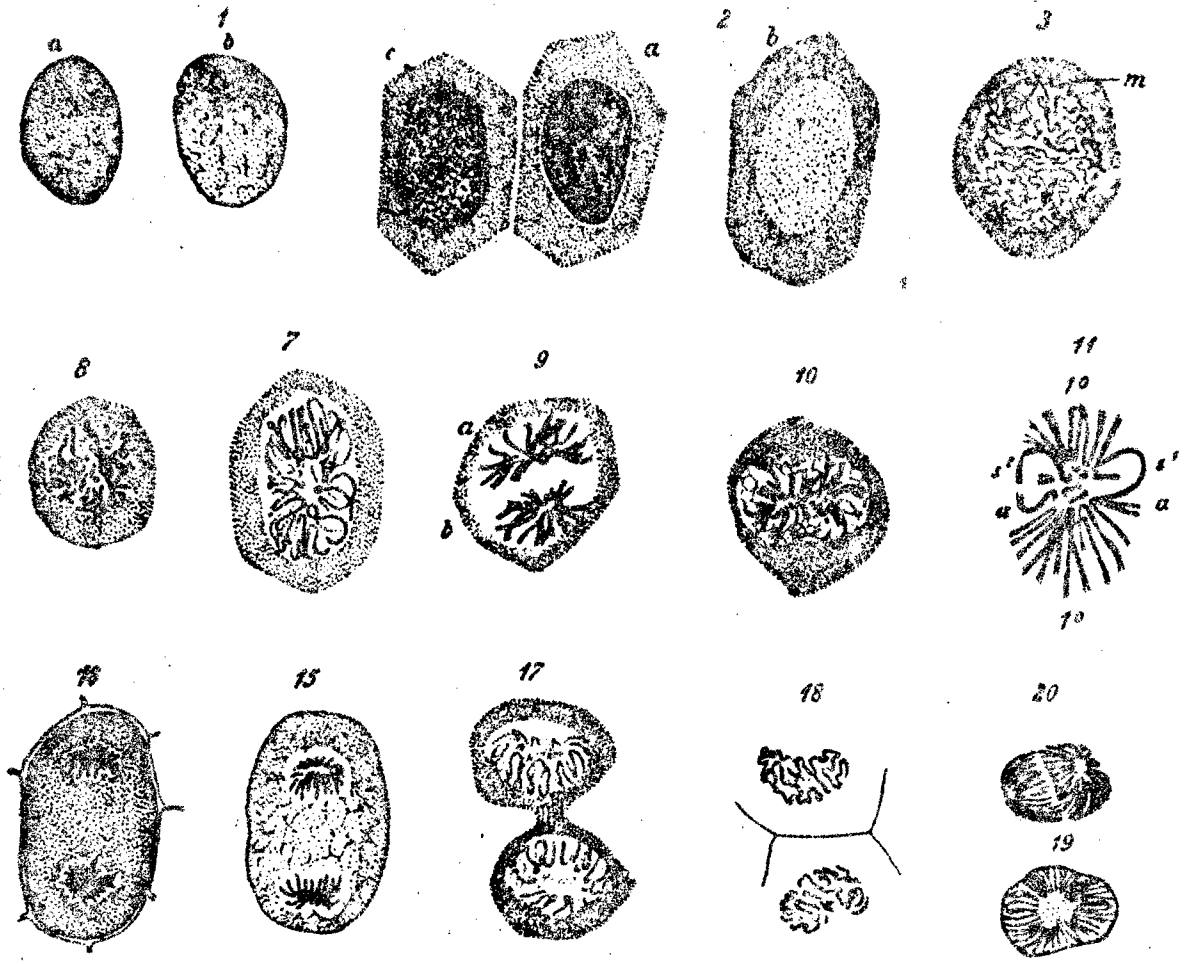

Fig. 8. Drawings of Dividing Cells of Larvae of the Salamander, from the Article of Flemming (1879B) Table 17.

The second part of his work appeared in the same journal in 1880. In it, Flemming took up various theoretical problems and gave much attention to terminology. In addition to the terms "direct" and "indirect" division, he introduced the term "karyokinesis" as a substitute for the term employed by Schleicher (Schleicher used the term "karyokinesis" to refer not to the entire process of division but to displacement and changes of the nucleus). For the "staining substance of the nucleus" he proposed the term "chromatin"; for the non-staining substance of the nucleus he used the term "achromatin." Accordingly, he introduced

the adjectives "chromatinic" and "achromatinic." The terms "maternal star (monaster)" and "daughter stars (diaster)" introduced by Fol to designate the rays in the protoplasm of the egg cell, were also adopted by Flemming for the corresponding nuclear figures. In the first section of his work, Flemming compares his ideas with the findings in the literature, especially with the three works of Peremezhko (Pages 165 to 168).

In 1882, there appeared the monograph of Flemming entitled "Cellular Substance, the Nucleus, and Cellular Division." In it he presented the general picture of division which lies at the basis of our contemporary ideas concerning karyokinesis. Indirect division was defined by Flemming as "a division of the nucleus-containing cell body into two or more parts, in which there is metamorphosis of the nucleus" (Page 194). This transformation is connected with the formation of the nuclear figure of division in which Flemming distinguishes two parts: chromatin and achromatin figures. In the process of division, Flemming distinguishes five states which correspond in general to the present-day four phases: the maternal nucleus (stroma, rest), the form of a skein (spireme), the form of a star (aster), the equatorial plate (metakinesis), the form of stars (diasters), the form of skeins (dispiremes), and daughter nuclei (stroma, rest).

For the figures of nuclear division Flemming proposes the term "mitosis," and for division which is not accompanied by "thready metamorphosis" he proposed the designation "amitotic division" (Page 376). The prophase is treated in the monograph of Flemming as a skein or tangle of chromatin threads, and he evidently believed that the chromosomes were joined by their ends.

In 1885, Rabl (1885) showed in the epidermis of tailed amphibia (urodeles) that, during prophase, chromosomes do not form continuous threads but are isolated one from another; in this process, the number of chromosomes in the nuclei of different tissues of one and the same object are identical. The notion of the individuality of chromosomes and of the presence of a constant number of them for each species during karyokinesis was later developed by Boveri (1888) in the second notebook of his "Studies of Cells."

Without going further into the ensuing development of the study of karyokinesis, let us nevertheless note certain terminological details. A number of the terms used at the present time were introduced, as we have seen, by Flemming (direct and indirect division, mitosis and amitosis, nuclear network, chromatin and achromatin, and stage of the skein); however, he was the first to introduce in their

modern sense the terms "maternal star" and "daughter stars," which were originally introduced by Fol in another sense. The terms "prophase," "metaphase," and "anaphase" were introduced by Strasburger (1884) (Pages 250, 260). His prophase almost corresponds to the contemporary meaning of the term, but he understood metaphase to refer to early anaphase in the modern sense. By anaphase, Strasburger understood the whole final period of division beginning with late anaphase and including the period which is now termed telophase. This latter term (telophase) was introduced by Heidenhain (1894, Page 524), but initially in a different sense from that recognized at the present time. Heidenhain used this term to designate the stage of "telokinesis," that is, the final displacements of the nucleus and centrosome at the end of mitosis. The term "interphase" was proposed by Lundegardh (1912, Page 21). In 1888, Waldeyer (1888) proposed the term "chromosome," and since that time this term has become firmly established in general usage.

This review indicates how unreliable are the majority of historical studies of the discovery of karyokinesis as listed at the beginning of this article. The discovery of karyokinesis is difficult to attribute to any single investigator. Essentially, karyokinesis was "discovered" in 1873 by Schneider, but this was of no influence on the subsequent development of the study of cell division, and Schneider himself did not understand the importance of the observations which he had made. The present development of the study of karyokinesis began with the work of I. D. Chistyakov, but his ideas were still very unclear and did not reveal the essence of this new phenomenon. The discovery and dissemination of knowledge concerning karyokinesis is the fruit of the collective efforts of a whole pleiad of scientists, among whom important roles were played by our Soviet scientists: I. D. Chistyakov, O. Butschli, Ye. Strasburger, B. I. Mayzel', P. I. Peremezhko, V. Flemming, K. Rabl' -- these are names of investigators to whom science is deeply indebted for the reformation of ideas concerning the division of cells -- the study of karyokinesis, which is one of the most important chapters in contemporary histology.

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CURRENT EVENTS

A Conference on Problems of Cytology, Histology, and Embryology, Held in Riga

L. G. Vol'fenzon and Yu. L. Goroshchenko

From the 10th to the 13th of December 1958, a conference was held in Riga on problems of cytology, histology and embryology, organized by the Institute of Biology and the Institute of Experimental Medicine of the Academy of Sciences of the Latvian SSR and by the Riga Medical Institute. This conference, the first since the end of the war, was an important event in the scientific life of the Baltic region, testifying to the fact that the development of morphologic studies has reached a very high level there.

In the work of the conference, in addition to scientists from Riga, scientific workers from all the Baltic Republics participated, as well as others from Belorussia, Moscow, and Leningrad. Thirty-nine reports were heard at the meetings of the conference, of which seven were read to plenary sessions, nine to the sections on cytology, eleven to the sections on histology, and twelve to the sections on embryology.

In the reports on cytology, a number of questions of modern cytochemistry were taken up, along with others on cytophysiology and the finer morphologic structure of the cell.

The first speaker at the first plenary session was G. O. Roskin (Moscow), who presented a report on problems in the cytodiagnostics of malignant tumors. The author discussed in detail the diagnostic importance of the reactions of normal and malignant changes of tissues with leucobasic methylene blue.

The nuclei of cells of normal tissues in both humans and animals are capable of staining a blue color with leucobasic methylene blue, whereas the nuclei of cells of the embryo, embryonic types of cells of the adult animal, and embryonic types of cells of malignant tumors, regardless of histogenesis, histologic nature, and species peculiarities of the tumor host, do not give a positive reaction with leucobasic methylene blue and remain unstained. The presence of colorless nuclei following this staining technique in certain tumors of the breast, in which pathohistologic studies fail to disclose malignant growth, apparently testifies to the presence of malignant change and may serve, therefore, as an early diagnostic sign. This reaction may unquestionably be of great assistance in analyzing different forms of

malignant tumors and, moreover, in the study of precancerous states under conditions in which the pathologic method is insufficient.

In the report of S. Ya. Zalkind (Moscow) data were presented on the comparative study of monolayer tissue cultures of the kidneys of Macacus rhesus monkeys, of the hearts of Macacus cynomolgus, and of two strains of malignant cells. The cultures were studied throughout their life cycles, from the moment of immersion in fresh nutritive medium until natural degeneration. The author found that each of these cultures is characterized by its own curve of mitotic activity. Morphologic analysis of the cultures demonstrated the presence in them of large numbers of amitoses. This suggests that cells of tissue cultures multiply not only mitotically but also amitotically.

In similar cultures L. P. Izakova (Moscow) studied the course of distribution of glycogen in the normal upon exposing the culture to the poliomyelitis virus. The author showed that there was a large amount of glycogen in the cells of all four cultures, with the type of deposition and the amount of the latter varying not only in different cultures but also within a single culture, depending on the physiologic state of the cells. The author showed, also, that there is a rhythmic character to the accumulation of glycogen by the cells over a period of time.

Two reports were devoted to problems of the functional histochemistry of nerve cells. In the report of Ya. A. Vinnikov and L. K. Titova (Leningrad), data were presented concerning the participation of a number of chemically active substances in the process of stimulation of receptor cells and neurons. These authors showed that, in the hair cells of the organ of Corti and in the neurons of the spinal ganglia, the amount, distribution, and activity of glycogen, phosphorylase, dehydrogenase, nucleic acid and the non-specific enzymes associated with them, SH-groups, and total proteins change, depending upon whether the cells are in a state of rest or in a state of stimulation following an adequate stimulus.

Hence, with the aid of cytochemical methods, it was possible to demonstrate the participation of processes of anaerobic glycogenolysis and oxidative respiration, the splitting of acetylcholine, and changes in the protein components (exposure of SH groups) upon stimulation, all such processes occurring in definite parts of the cell.

The purpose of the studies of L. B. Levinson (Moscow) was to investigate the connection between structure, chemical composition, and the specific functions of nerve cells. The author showed that, in motor and sensory cells of chick and rat embryos at critical moments of development of the

specific functions, there are changes in the concentration of RNA, in the relative amount of histodene in the proteins, and in the distribution of thiol groups in the cell structures. In the nerve cells of chick embryos, there are changes also in the reaction to alkaline phosphatase and Vitamin C, as well as in the shape of the mitochondria - organoids associated with the basic enzymes of the cell systems.

It was further shown that the amount of RNA in ganglionic motor cells of the fly Callyphora is reduced in proportion to the strengthening of motor function, and remains high in non-flying flies and in flies in which the wings were removed prior to the onset of hatching. Similar results were seen in the retina of the eye: under the influences of light stimulation the concentration of RNA increased in cells, and with an intensification of stimulation sufficient to lead to exhaustion, there was a decrease in the concentration of RNA.

Hence, the author was able to show that, in the process of development of specific functions of nerve cells in the embryonic stages of development, and also in the actively working nerve cells of the adult animal, there are changes in chemical composition.

Interesting reports devoted to the participation of nuclei in the morphology of secretion of cells were presented by the associates of K. F. Bogoyavlenskiy (Riga). In a report on "The Morphology of Secretion in Cells of the Corpus Luteum," A. R. Dalmane (Riga) described the formation and growth of vacuoles both in nucleoli and in the immediately contiguous karyoplasm of the nucleus of cells in the corpus luteum of the white rat. She also described the mechanism of evacuation of the contents of these vacuoles into the cytoplasm by means of dissolution of the nuclear membrane and direct secretion of RNA of the nucleoli into the cytoplasm.

This same problem was taken up in the report of I. D. Chernobayeva (Riga) on "Morphologic changes in cell nuclei of the exocrine portion of the pancreas during secretion." The onset of formation of secretory granules in the cells of the pancreas is preceded by an increase in the amount of RNA in the cytoplasm and the appearance in it of a considerable number of vacuoles. In proportion to the accumulation of secretion, the content of RNA in the cytoplasm decreases and the number of vacuoles also decreases. The author describes a further increase in the size of the nucleus with the onset of formation of secretions, the appearance in it of a large number of vacuoles, and their irruption through the nuclear membrane into the cytoplasm. The RNA of the nucleolus during the period of accumulation of zymogen in the cell is gradually replaced by vacuoles, the contents of which are

also secreted into the cytoplasm. Disruptions of nervous regulation (vagotomy) and the release of secretin, pilocarpine, and ephedrine cause an intensification of these changes and a displacement of the phases of secretion in time.

Kh. Ya. Puzhaka (Riga) presented findings on the influence on mitotic activity of adrenalin and pilocarpine under conditions of complete absence of neurohumoral regulation. In isolated corneas of white rats kept in physiologic saline solution, the number of mitoses gradually decreases. The addition of adrenalin accelerates this process, whereas the addition of pilocarpine elicits an intensification of proliferation as compared with controls. The author concludes that the influence on mitotic activity of adrenalin and pilocarpine in isolated tissue is analagous to the effect of these substances on cell division in the organism.

The report of L. G. Vol'fenzon (Leningrad) was devoted to a study of the effects of novocain on a number of tissues not of the nerve type. He showed that novocain, in concentrations used for purposes of local anesthesia, causes a reversible injury of mesothelial and mast cells of the rat peritoneum.

Ya. T. Tsinovskiy (Riga) reported on studies of cellular elements participating in the formation of the sex cells of certain beetles and sawflies. The author studied the initial period of gametogenesis in these objects and showed that an active role in this process is played by apical cells, which support and provide nutrition to the primary ovogonia and spermatogonia.

Yu. K. Bogoyavlenskiy (Moscow), studying the finer structure of the cuticle with the aid of the electron microscope, demonstrated in its composition the presence of two layers in addition to the six already known and also described the finer structure of each layer.

In the report of V. F. Mashanskiy, U. L. Goroshchenko, and T. N. Mosevich (Leningrad) devoted to an electron microscopic study of the superficial layer of spermatocytes of the mite, a description was given of the formation and finer structure of this layer, and also of the development of special fibrils on the surface of the prospermia which apparently exercise a supportive function.

In the report of G. U. Abraytis (Kaunas), read at a plenary session, extensive pathological and anatomical material was used to investigate the changes of the gastric mucosa in the region of the lesser curvature in the vicinity of the pylorus. The author showed that the number and size of pyloric glands, the thickness of the connective tissue layers between them, and the thickness of the mucosa of this part of the stomach change with age.

In the histology section, reports were heard on the innervation of different organs in the normal and under different pathologic conditions: F. B. Kheyman (Minsk) gave a description of the innervation of the urinary bladder; A. S. Vitkus (Kaunas) studied in detail the intraorgan nerves of the spleen; P. I. Lobko (Minsk) read a report on the participation of cell processes of spinal ganglia in the formation of the celiac plexus; Ye. N. Chayka (Minsk) described the growth of cells and fibers of the pelvic plexus during chronic pathologic processes.

In a report entitled "The Problem of the Division of Nerve Cells," S. A. Agureykin (Riga), on the basis of data from the literature and of his own observations (a morphologic study of cells of the superior cervical ganglion of the rabbit after transection of the preganglionic nerve fibers), concluded that the prevailing point of view concerning the constancy of the quantitative composition of neurons in the organism is correct.

O. N. Vinogradova (Riga) studied the structure of synapses in the gastrointestinal tract in different species of vertebrate animals and following vagotomy. She showed that the contact apparatus of the autonomic nervous system of vertebrates is distinguished by a diversity of morphologic structures and exhibits species differences.

Reports were also heard from Kh. V. Kubar (Tartu) concerning variability in the volume of the glandular tissue of the oviduct in domestic birds; from S. Aul' (Tartu) concerning structural and functional changes in the sympathectomized uterus of the rabbit; and from A. T. Galeniyetse (Riga) concerning changes in the blood, bone marrow, and connective tissues under the influence of an extract of ascarids.

P. Shevitskis (Vilnius) presented a report at a plenary meeting on embryologic findings concerning the problem of regeneration in the animal world.

At the final plenary session, interesting reports were given on the formative role of the mesenchyme in the growth of the human fetus by P. Ya. Gerke (Riga). Giving numerous examples, the speaker showed that the embryonic mesenchyme during the growth of the fetus is the first independent tissue to be distributed outside the embryonic layers. It participates in the formation of the embryo as a sort of tissue medium, facilitating the growth and differentiation of parts of organs and their mutual replacement. Later, with the cessation of differentiation, the embryonic mesenchyme disappears, giving place to the cellular elements characteristic of the definitive structure of the organ.

The majority of reports evoked a lively exchange of

opinions and sometimes a sharp discussion, despite the fact that the absence of printed summaries in some cases complicated the problem of orientation with respect to the content of the reports. The work of the conference proceded in a business-like and friendly atmosphere, and demonstrated the fruitfulness of this type of small conference of workers in various but related specialties from different cities and republics.

Books on Cytology and Related Disciplines the Publication of Which is Projected by the Publishing House of the Academy of Sciences USSR for 1959

Nasonov, D. N. "The Local Reaction of Protoplasm and Spreading Stimulation," (Institute of Cytology of the Academy of Sciences USSR), 38 quires, 2000 copies, price 28 rubles. Publication date in the second quarter.

Kedrovskiy, B. V. "The Cytology of Protein Syntheses in the Living Cells" (Institute of Animal Morphology of the Academy of Sciences USSR), 30 quires, 2500 copies, price 23 rubles. Publication date in the first quarter.

Lopashov, G. V. "The Mechanism of Development of the Anlage of the Eye During Embryogenesis in Vertebrate Animals" (Institute of Animal Morphology of the Academy of Sciences USSR), 13.5 quires, 2000 copies, price 10 rubles. Publication date in the second quarter.

"Problems of Photosynthesis" Reports at the Second All-Union Conference on Photosynthesis held in Moscow in 1957, Section on Biologic Sciences of the Academy of Sciences USSR (The Biology and Soil Faculty of Moscow State University), 50 quires, 5000 copies, price 37 rubles. Publication date in the second quarter.

Ratner, Ye. N. "The Nutrition of Plants and the Life Activity of Their Root Systems" (Institute of Plant Physiology of the Academy of Sciences USSR), the Timiryazev Lectures, No 16, 5 quires, 5000 copies, price 4 rubles. Publication date in the first quarter.

Sabanin, D. A. "The Physiology of Plant Growth" (Institute of Plant Physiology of the Academy of Sciences USSR), 12 quires, 4000 copies, price 8 rubles, 40 kopecks. Publication date in the first quarter.

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